Modification of tumor growth by blood transfusion and perioperative procedures

A study in rats

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MODIFICATION OF TUMOR GROWTH BY BLOOD TRANSFUSION AND PERIOPERATIVE PROCEDURES

A study in rats

BEINVLOEDING VAN TUMOR GROEI DOOR BLOED TRANSFUSIES EN PEROPERATIEVE PROCEDURES

Een onderzoek bij ratten

PROEFSCHRIFT

Ter verkrijging van de graad van Doctor in de Geneeskunde
aan de Erasmus Universiteit Rotterdam
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Door

Sandeep Kumar Singh

Geboren te Etah
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To my parents,
Sudhir, Alka, Anil and Anjali

Believe nothing
Merely because you have been told it
Or because it is traditional
Or because you yourself imagined it
Do not believe what your teacher tells you
merely out of respect for the teacher
But whatever after due examination and analysis
you find to be conclusive to the good
the benefit,
the welfare of all beings,
that doctrine believe and cling to,
and take it as your guide.

Gautama Buddha. (563-483 BC).

CONTENTS

CHAPTER 1

INTRODUCTION

A blood transfusion is a transplantation of all or part of the blood cells in the peripheral blood. The survival of grafted organ transplantation has been shown to be prolonged following blood transfusion. The mechanism is thought to be immunological in nature. The possibility of a relationship between blood transfusion and cancer growth has initiated numerous studies, investigating various aspects of transfusions and cancer. Nevertheless, the relative importance of this phenomenon is still matter for discussion. Certain aspects of cancer and blood transfusions relevant to the experiments reported in this thesis are briefly discussed below.

1.1. Tumor Immunology:

Cancer is characterized by the uncontrolled and disorderly multiplication of abnormal cells. Although the precise mechanism of this malignant process is not known, various factors are recognized as being able to initiate that process. Chemicals, radiation, genetic factors, repeated trauma, and most recently, viruses have been proven to cause or to be strongly associated with certain forms of cancer. The concept that cancer is a disease sequential to immunological factors dates back to the turn of the century. At the begining of this century Loeb[1] demonstrated rejection of incompatible tumor grafts. In the 1940's, Snell[2] pointed out role of immunogenicity of tissue in studies of transplantable neoplasia to hosts of different histocompatibility constitutions. Lymphocyte infiltration into the malignant tissue, unrelated to tissue necrosis, has been correlated with a better prognosis. This is indicative of an immunological type of host resistance[3]. A lymphoid predominance type of Hodgkin's disease is indicative of good prognosis, while the lymphoid depleted type of this tumor bears an unfavourable prognosis. A far higher incidence of malignant tumors, than would be expected when compared with the normal background population, is observed in kidney transplant recipients, who receive immunosuppressive drugs continuously[4]. Partial or complete regression of lymphoma or kaposi's sarcoma is reported following reduction or cessation of immunosuppressive therapy[5,6]. These observations support the concept of a relationship between cancer and immunological factors.

In a bird's eye view, a summary of immunological factors of host and tumor is presented below. This is followed by an evaluation of the evidence in support of and against a role of immunological factors in tumor genesis and progression. It should be stressed that no attempt is made to provide an exhaustive review of

The initiation of cancer development and most stages of cancer progression are most likely to involve changes at the genetic(DNA) level, as mitosis of a cancer cell results in an another malignant cell. Changes in DNA can result in different proteins being produced leading to changes in cell membranes. This in turn may alter the uptake and release of materials, and alter the adhesiveness and cohesiveness, so that invasion and metastases result[7]. Malignant transformation may also be accompanied by phenotypic changes in the involved cells, including the loss of normal antigenic cell surface components, gain of neoantigens and gain of new antigens. Altered antigens, capable of evoking the rejection of tumor cells in pre-immunized syngeneic hosts are termed "Tumor Associated Transplantation Antigens"(TATA). The presence of TATA is supported by results of Foley et al[8] who showed the development of immunity in mice, which inhibits the growth of the reimplanted tumor tissue after the removal of the primary tumor. Klein et al[9] have demonstrated that immunization of syngeneic mice with irradiated tumor cells from a primary sarcoma, leads to immunity to a same line of neoplasma, but not to any other of the 12 sarcoma cell lines obtained from the same mouse strain.

Various types of "Tumor Associated Antigens" (TAA) are found to be expressed by tumor cells with respect to etiological factors[10,11]:

a) Virus induced tumors

Tumor associated antigens induced by a virus are the same in all tumors if induced by the same virus in different animals of the same strain, or of different strains and even of different species. These antigens are determined by the viral genome. Two types of virus-specific antigens have been distinguished, according to the location in the cell.

1) Intra cellular antigens: These antigens can be located in the nucleus or cytoplasm of a viral infected cell and are encoded in viral genetic material. These antigens are shared by cells infected with a particular virus, irrespective of whether or not this viral infection propagates into oncogenesis. The role of these antigens in tumor immunity is not known.

2) Cell surface antigens: These are also viral gene products, coded in viral DNA or RNA. These antigens are capable of acting as transplantation antigens, **i.e.:** hosts inoculated with tumor cells displaying surface antigens will develop increased resistance to subsequent challenge with the same tumor cells.

b) Chemically and Physically Induced Tumors

Antigens associated with these tumors vary from one tumor to another, even when the tumors are induced by the same carcinogen in the same strain or even in the same host. This polymorphism in antigen expression is unexplained, but may reflect the array of DNA changes induced by these carcinogens.

c) Embryonic Antigens

These are an expression or products of antigens normally only found in fetal tissue. They are also detected in very small amounts in non-malignant adult cells **e.g.:** α-fetoprotein; carcino-embryonic antigens(CEA). These oncofetal antigens are not strictly tumor associated but some of them are capable of functioning as TATAs eliciting rejection of tumor in immunized hosts; this indicates a varying degree of effectiveness of these antigens in tumor rejection.

d) Spontaneous Tumors

Spontaneous tumors are tumors arising in circumstances where no oncogenic agent has been proven to be involved. These tumors usually possess very weak or no detectable TAAs, but many express fetal antigens on the cell surface. Since etiological agents of tumors arising in man are often unknown, many of them may belong to this type of tumor.

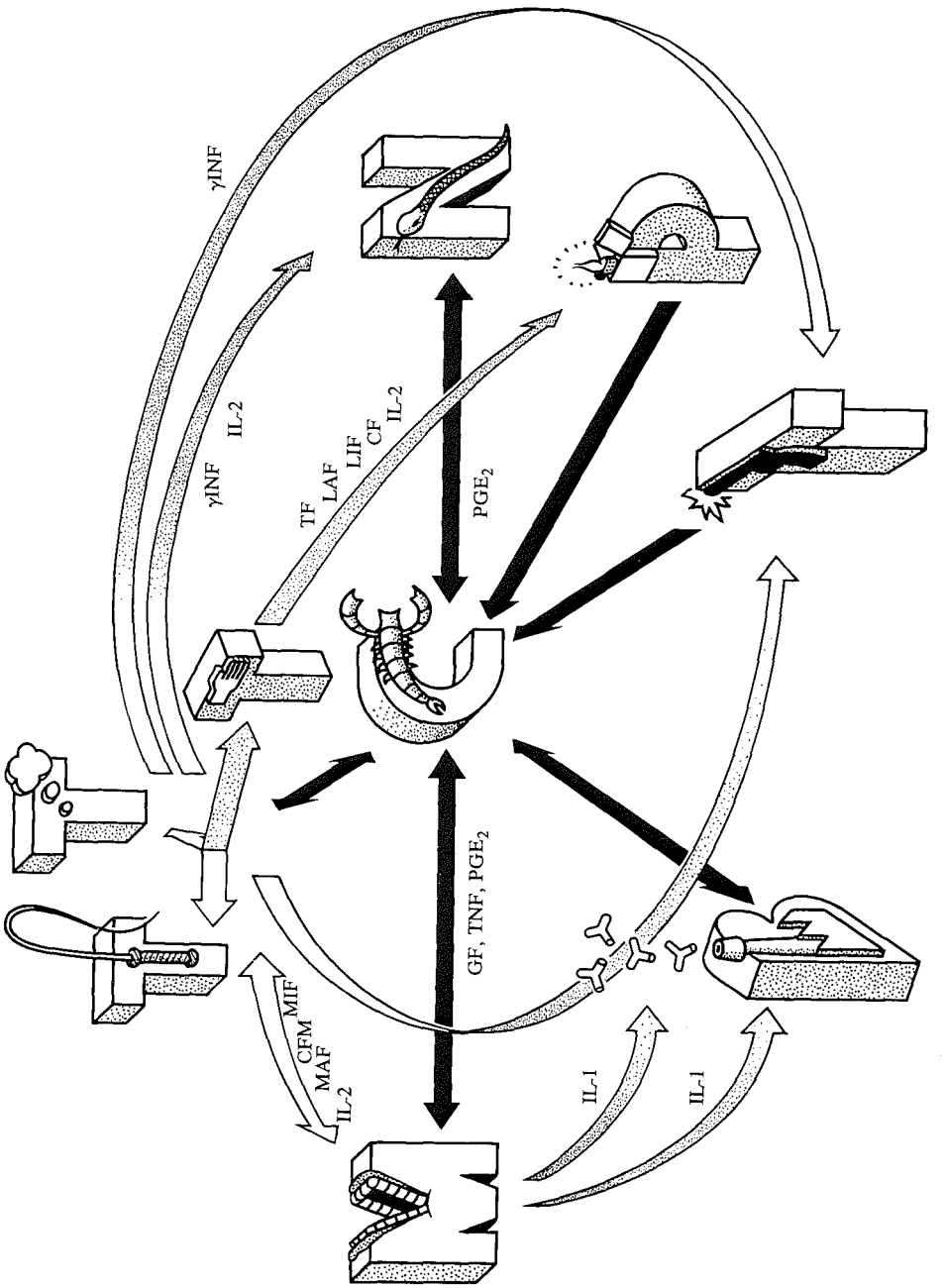
The changes in antigenic phenotype of tumor cells can elicit an immune response against the tumor antigen. These responses are complex and have not yet been fully elucidated. However, several effector mechanisms have been implicated in this response **i.e.:** T-lymphocytes; specific and non-specific cytotoxic macrophages; Natural Killer(NK) cell; specific antibodies; natural cytotoxic antibodies; and Killer cells[10-12]. The specific properties of these mechanisms are summarized below (Figure 1):

T-Lymphocytes

During an immune reaction the T-lymphocyte can present itself as a helper T-cell(T_h), suppressor T-cell(T_s), and cytotoxic T-cell(CTL). Activated T-helper lymphocytes can produce a spectrum of activities and subsequently may play a pivotal role in the modulation of the immune reaction. Many of the lymphokins produced by these T-cells participate in the activation of other effector mechanisms implicated in the response. **e.g.:**

* The migration inhibiting factor(MIF), the macrophage activating factor(MAF) and the chemotactic factor for macrophages(CFM) can lead to the arrest of macrophages at the tumor site. They can further initiate phagocytosis by macrophages and lead to the development of "activated macrophages".
* The chemotactic factor(CF) and the leukocyte inhibiting factor(LIF) influence the recruitment and activation of neutrophils, capable of lysing tumor cells.
* The transfer factor(TF) and the mitogenic factors (of which interleukins 1 and 2 are the most studied) can exert an effect on other lymphocytes.
* Lymphotoxins which are capable of lysing tumor cells by direct interaction.
* Interferons (α, β and γ-interferons): various effects are attributed to these with regard to modulation of immune responses. But there is still no consensus regarding their overall role in tumor immunology.

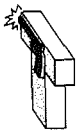
Figure 1: Relationship between cancer and immunology.



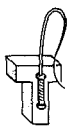
Legend to Fig. 1:


Cancer cell


T-helper cell


T-killer cell

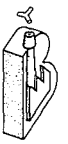

T-memory cell


T-suppressor cell


Macrophage


Polymorphonuclear leukocyte


Natural killer cell


B-lymphocyte

IL-1 and 2	:	Interleukin 1 and 2	TF	:	Transfer factor
GF	:	Growth factor	TNF	:	Tumor necrosis factor
γINF	:	γ-Interferon	PGE_2	:	Prostaglandin E_2
MAF	:	Macrophage activating factor	CF	:	Chemotactic factor
MIF	:	Migration inhibiting factor	LIF	:	Leukocyte inhibiting factor
CFM	:	Chemotactic factor for macrophages	LAF	:	Leukocyte activating factor

T-suppressor cells are capable of suppressing the immune response elicited by the host.

Certain T-lymphocytes recognize tumor associated antigens in association with major histocompatibility complex(MHC) products and then differentiate into cells endowed with specific cytotoxic activity towards cells with those specific tumor antigens. These cells are called Cytotoxic T-lymphocytes(CTL), and may be important in the growth control of antigenic tumors.

Macrophages

Macrophages demonstrate a variety of functions in reaction to tumor cells. They play a part in the processing and presentation of antigens. This is a crucial factor with respect to antigenic determinant presentation, in the process of triggering T- and B-lymphocytes. Triggering can result in suppression or activation of different subsets of T-lymphocytes. MIF, MAF and CFM and other lymphokines produced by T-lymphocytes can result in "activated" macrophages.

These cells express an increased non-specific antimicrobial activated and potent tumoricidal activity. They are also implicated in releasing factors capable of modulating tumor growth. A Tumor necrosis factor(TNF), produced by macrophages, can contribute to the killing of tumor cells. In contrast, they are also implicated in releasing of certain growth factors that can stimulate the growth of tumor cells. Furthermore, these cells are capable of directly activating complement C_3, which in turn amplifies the general inflammatory response at the tumor site.

Natural Killer Cells

These are non-adherent, non-phagocytic cells with spontaneous cytotoxicity which can efficiently lyse virus-infected, neoplastic and immature cell types of normal tissue. The target structures recognized by NK cells in observed cytotoxicity do not show MHC related restriction. NK cell activity is subject to both stimulatory regulation, via IFN and IL-2, and suppressive regulation, via Prostaglandins E_2 (PGE_2) produced by macrophages, T-lymphocytes and neoplastic cells. The importance of NK cells in opposing tumor growth is as yet uncertain.

B-Lymphocytes

Antigenic stimulation results in proliferation and antibody formation by B-lymphocytes. This response is further modulated by lymphokins produced by different T-lymphocyte subsets. The antibodies produced can demonstrate direct lytic properties to tumor cells or recruit other cells **e.g.:** macrophages and killer cells, which in turn can show tumoricidal properties. Furthermore, they may subvert the cellular immune response to a tumor by forming soluble antigen-antibody complexes or masking tumor antigens.

Killer Cells

These cells are able to destroy target cells coated with antibodies directed against cell surface antigens. Cytotoxicity of these cells is induced by Ig G antibodies and is independent of the complement system. This phenomenon is known as "antibody dependent cell mediated cytotoxicity "(ADCC).

The importance of the factors mentioned above is only relative. New antigens are not exclusive to malignant tumors; they can also be observed in preneoplastic tissues. Furthermore, very often no antigen can be demonstrated on the majority of spontaneous tumors. Interpretation of data on tumoricidal effector mechanisms is difficult because we are dealing with data from very heterogeneous tumor populations like transplantable or spontaneous tumors, different degrees of antigenicity, and a difference in the biology of various tumor types[13]. It cannot be stressed enough that cells like lymphocytes and macrophages

infiltrating into a tumor are not only cytotoxic effector cells. Both types of cells in a tumor may participate in the development of the immune reaction (**e.g.:** helper T-cells and macrophages presenting antigens); they may be effector cells (CTL and cytotoxic macrophages) or suppressor cells (suppressor T-cells and macrophages). Thus the mere presence of macrophages or lymphocytes in a tumor does not mean that these cells are tumoricidal cells. Furthermore the tumor itself may play a very important role in modulation of the immune response[14]. Tumor antigens can lead to either rejection or enhancement of tumor growth, depending on the form of interaction with the immune apparatus[15,16]. Formation of soluble tumor antigen antibody complexes or masking of tumor antigens by antibody may lead to growth of tumor cells. In addition, tumor cells may release factors **e.g.:** PGE_2, which could lead to suppression of NK and Killer cell activity.

Over the course of time, two hypotheses have emerged to explain how organisms prevent tumor growth. The basis of these hypotheses is formed by the assumption that the daily turnover of cells in the body results in many mutated cells, a certain percentage of which are malignant in nature. This assumption stipulates that innumerable tumors could develop. The fact is that tumors do not occur frequently during a lifetime[17], and furthermore most major mutations are known to be incompatible with the survival of the cell.
The two hypotheses are:

1) The immune surveillance theory

This theory attributes a major function to T-lymphocytes: **i.e.** to recognize the mutant cells and to destroy them or inhibit their growth. The T-lymphocyte response is implicated to be induced by and directed against the tumor antigens.

2) The natural resistance theory

This theory attributes resistance to tumor growth to be the result of NK cells, K-cells, granulocytes and macrophages, which are not induced or activated by antigens on tumor cells.

The assumption of the occurrence of de novo tumor cells, based on these hypotheses, necessitates a very potent surveillance or natural resistance capable of killing these cells, during a very long period. The peak tumor incidence in aged people could be explained by the decreased capacity of the immune system with passage of time, giving one of the multitude of tumor cells the opportunity to "sneak" through the two postulated mechanisms.

Recent evaluation of evidence has, however increasingly produced arguments contradicting the validity of the two hypotheses[18]. Some of the evidence is listed below:

* The incidence of spontaneous tumors in T-cell deficient nude mice is not higher than in immuno-competent animals[19,20].
* Most tumors that metastasize in normal immuno-competent animals fail to do so in nude mice[21].
* Some murine melanoma cells that are resistant to lysis by T-lymphocytes are less metastatic than cells that are susceptible to lysis by T-cells[22].
* It is mainly tumors of the skin and solid lymphomas which develop in immuno-suppressed animals and human patients, whereas according to the basic assumption of the hypothesis tumors should arise in all tissues that divide rapidly[4,23,24,25].
* Tumor growth rate in immunologically privileged sites is not faster, but often slower, than that of neoplasms in immunologically exposed sites[26].
* Spontaneous tumors in both man and animals frequently lack detectable antigens[27].
* Mice bred under sterile conditions, do not have natural cytotoxic macrophages. According to mutation frequency such a mouse would develop many tumors. This is not the case[28].
* In mice, NK cell activity is not present before birth or after one year of age. The absence of NK cells, however, does not correlate with increased tumor incidence.
* Not every type of tumor is found to be sensitive to natural cytotoxic antibodies, NK cells or macrophages which would disqualify these cells as efficient tools of natural resistance to many of the arising tumor cells[29].

The above mentioned arguments indicate the failure of each anti-tumor system, to kill newly arising tumor cells. However complementation and mutual compensation of these systems could be more important and should be considered. Furthermore, their effectiveness could be better evaluated if we knew exactly the number of de novo arising tumor cells that need to be killed and the variety of immunological mechanisms available. In short, we still do not know enough, and there is much more to be learned about fundamental immunological mechanisms. Studies on the role of host immunity in cancer metastasis have also yielded many contradictory results [13,15,30,31]. In many transplantable tumor systems, depression of host immunity increases the incidence of experimental and spontaneous metastases; in other tumor systems, depression of immunological reactivity decreases, or even prevents, metastases or has no influence on the growth of a local or disseminated tumor. The basis for these discrepancies could be the different etiologies of tumors and the different species employed in these studies. But this further illustrates the many factors playing a role in the final outcome of tumor growth. Thus, host immunological factors, in interplay with tumor cell properties, seem to be involved in the progression of tumors but other unidentified factors may be of even greater importance.

Yet another example of the complex relationship of immune apparatus and tumor growth is demonstrated by the great variety of approaches attempted at **Immunotherapy i.e.:**

* Non-specific immunization: This employs several reagents **e.g.:** BCG and C.Parvum, which are capable of general stimulation of the lympho-reticular system.
* Active immunization: involves vaccination against the assumed tumor specific antigens. This entails the use of autologous tumor cells that are rendered incapable of growth by irradiation or treatment with mitomycin C, to immunize the host.
* Passive immunotherapy: entails transfer of anti tumor antibodies to cancer patients in order to cause tumor regression or prevent tumor recurrence.
* Adoptive immunotherapy: involves the transfer of immunocompetence or tumor immunity via leukocytes. The latest form of this therapy is lymphokine activated killer cells (LAK cells) **i.e.** host leukocytes are activated by lymphokines, notably interleukin-2 (IL-2) in vitro, and then reinjected into the host in the belief that their activation has rendered them more effecient in their tumoricidal function. This procedure has produced most promising results in the fight against cancer growth compared to any of the possiblities mentioned above[32].
* Biological response modifiers: these are substances, **e.g.:** Poly A-poly U and ABPP, capable of inducing the production of various lymphokines or augmenting immune responses in order to control tumor growth.

1.2. Blood transfusions and Immunology

Throughout human history, "blood" has been symbolized as possessing many mystic and omnipotent powers. In 1492, Pope Innocent VIII was the first human to receive a blood transfusion. The Pope and the three blood donors succumbed during the procedure; no doubt, evil spirits were blamed at the time. Increasing knowledge of blood groups, blood chemistry and technical advances has led to ever increasing numbers of blood transfusions being administered in the modern practice of medicine. The possibility of successful blood transfusions has resulted in the success of many surgical procedures and improved patient care. Coincident with greater clinical use of blood transfusions, awareness of the possibility of associated morbidity has increased.
The present known risks of using allogeneic blood transfusions include:

* mismatched transfusion due to technical errors of typing contributing to grave complications[33];
* isoimmunization to antigens present in the transfusate[34-36];
* risk of hemolytic, febrile reaction;

* graft vs host reaction[34-36];
* transfusion related acute lung injury[37];
* anaphylaxis[38];
* transfusion transmitted acquired immuno-deficiency syndrome(AIDS)[39];
* transmission of other diseases **e.g.:** hepatitis, syphilis, malaria, CMV virus, Epstein-Barr virus[40,41].

In contrast, blood transfusions have also been shown to have a beneficial effect on kidney transplant survival[42,43]. However, the mechanism responsible for this effect has not been fully elucidated. Evidence suggestive of a non-specific immunosuppression mediated by suppressor cells has been presented, while on the other hand evidence in support of a specific unresponsiveness mediated by anti-idiotypic antibodies which inactivate particular cell clones also exists[44,45]. Theories implicating "clonal" deletion of T-cells and selection of low responder patients as a result of blood transfusion have been put forward as a possible explanation[46,47]. In 1981, by proposing an analogy between tumor associated antigens and major histocompatibility antigens, which play a major role in transplant outcome, Gantt[48] speculated that the induction of a state of immune tolerance by blood transfusions could, via non-specific immune suppression, have an effect on tumor growth. In the event of this being true, it would be a point of extreme importance because a considerable number of cancer patients are anemic or become anemic due to blood loss at operation, and thus are regularly transfused. One of the greatest problems in the management of malignant disease is the recurrence of metastases. The above phenomenon could in fact augment the incidence of metastases, resulting in a poor prognosis for cancer patients. For these reasons, an effort was made to test the validity of this speculation.

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CHAPTER 2

RATIONALE AND OBJECTIVES OF THE EXPERIMENTS PERFORMED.

Gantt's[1] initial proposal of a possible relationship between blood transfusion and prognosis of surgically treated cancer patients has stimulated many reports of experimental and retrospective clinical studies. A prospective study has also been carried out, which looked at possible modulation of tumor growth in relation to blood transfusions. Jeekel et al and Francis et al[23,24] were the first to report data relating to this speculation. Retrospective reports dealing with patients with breast, lung, kidney, colon-rectum, cervix carcinomas and soft tissue sarcomas, undergoing "curative" operations produced a varied picture of the effects of blood transfusions(Table I). Burrows et al[2] reported clinical data providing credence to the speculation of Gantt[1]. In a retrospective study of 122 patients who had undergone curative surgery for colon-rectum carcinomas, they demonstrated significantly lower disease free survival rates for transfused as compared to non-transfused patients, the difference being apparent as early as 6-12 months after surgery. This difference persisted when variables of location, tumor stage or post-operative adjuvant therapy were taken into account. Blumberg et al and others have confirmed this adverse outcome of patients with colon-rectum carcinoma following blood transfusion[3-5]. Furthermore, they reported an association between poor prognosis and higher age, lower hemoglobin level, longer duration of the operation and the tumor site. However, several other retrospective studies, analysing their data in a similar manner, were unable to demonstrate an association between detrimental survival rates or disease free survival rates and administration of blood transfusions[6-10]. In an prospective study, Frankish et al[11] were also unable to confirm an association between perioperative blood transfusion and recurrence of colon-rectum cancer within a three year follow-up period.

Similarly, retrospective data analysis of patients with breast cancer has resulted in contradictory reports[12-15]. Nowak, Foster and Miller et al. were unable to find any relationship between blood transfusions and prognosis of women undergoing surgery for breast cancer[13-15]. Bickel et al[6], looking at survival rates, did find a relationship between poor prognosis and a group of breast cancer patients receiving blood transfusions. However, of the 68 patients in the group analysed, only 17 received blood transfusions. In their exhaustive analysis of 169 patients, Tartter et al[12] found a significantly lower cumulative 5-year disease free survival rate for patients receiving transfusions (51%) compared to patients who did not receive blood (65%). However, a markedly lower admission hemoglobin value and greater blood loss during surgery were found among patients receiving transfusions. Furthermore, a significantly worse prognosis was found for patients who had lost a considerable amount of blood as compared to those who had lost less blood during surgery.

Evidence that transfusions affect the prognosis of patients undergoing surgery

Table 1: Effect of blood transfusion on prognosis after operation for a tumor.

Reference		Tumor site	DFSR/SR*	Transfusion** effect
Burrows et al	(2) 1982	colo-rectum	DFSR	+
Blumberg et al	(3) 1985	colo-rectum	DFSR	+
Foster et al	(4) 1985	colo-rectum	SR	+
Parrott et al	(5) 1986	colo-rectum	DFSR/SR	+
Bickel et al	(6) 1985	colo-rectum	SR	−
Nathanson et al	(7) 1985	colo-rectum	SR	−
Francis et al	(8) 1985	colo-rectum	DFSR	−
Ota et al	(9) 1985	colo-rectum	DFSR/SR	−
Weiden et al	(10) 1984	colo-rectum	DFSR	−
Frankish et al	(11) 1985	colo-rectum	DFSR	−
Tartter et al	(12) 1985	breast	DFSR	+
Foster et al	(13) 1984	breast	SR	−
Miller et al	(14) 1984	breast	DFSR	−
Nowak et al	(15) 1984	breast	DFSR	−
Bickel et al	(6) 1985	breast	SR	+
Tartter et al	(16) 1984	lung	DFSR	+
Hyman et al	(17) 1985	lung	SR	+
Bickel et al	(6) 1985	lung	SR	+
Manyonda et al	(19) 1986	renal	SR	−
Moffat et al	(20) 1985	renal	SR	+
Rosenberg et al	(21) 1985	sarcoma	SR	+
Blumberg et al	(22) 1985	cervix	DFSR	+
Blumberg et al	(22) 1985	prostate	DFSR	+

*DFSR: Disease free survival rate.
SR: Survival rate.
** +: Detrimental effect
−: No effect.

for lung cancer has been supplied by three retrospective studies. Tartter et al[16] analysed disease free survival rate, while Hyman et al[17] and Bickel et al[6] considered survival rates of the patients, and indicated that a blood transfusion was a significant prognostic factor for lung cancer patients undergoing surgical resection.

Rosenberg et al[21], in his series of patients with high grade soft tissue sarcomas and Blumberg et al[22], with data on cervix cancer, also reported a poorer prognosis related to patients receiving blood transfusions.

In accordance with the clinical retrospective data reported above, similar contradictory results are reported from experimental animal studies[23-27]. Using a chemically induced sarcoma, Francis et al[23] found a more rapid growth of tumor transplants in rats that had received transfusions of allogeneic blood than in rats that had not been transfused or had received syngeneic blood. In a different model, conflicting results are reported by Jeekel et al[24], who injected rats intravenously with a basal cell carcinoma or an adeno-carcinoma cells. More pulmonary nodules were found if animals had not received allogeneic transfusions. In yet another tumor-host model, Oikawa et al[25] also found inhibition of a transplantable fibrosarcoma following allogeneic blood or leukocyte transfusion. Zeller et al[27] employing five different models, were unable to find differences in take rate, induction time, incidence and growth rate of tumors following allogeneic blood transfusion. The differences in results of these studies may be related to differences in animal blood donors, experimental tumors, routes of inoculation and methods of assessment of tumor progression.

Numerous plausible explanations for the conflicting results of retrospective studies can be presented. Retrospective clinical studies are always restrictive in the manner in which they can be analysed, as one lacks the control of obtaining data which one may consider relevant. The inclusion and exclusion criteria are particularly important and may lead to discrepancies. In spite of thorough statistical analysis of the data, the possibility of a mathematical artefarct always persists. The parameters used in a study can also effect the conclusions reached. In studies with rate of recurrence as parameter of prognosis, the frequency of follow up and the intensity of investigation can alter the time of first recognition of recurrence; thus, studies not conducted under a rigid protocol must be regarded with circumspection. Since surgically treated patients are considered in these studies, and surgeon-related variables have been shown to be of great influence on clinical results obtained[28], this may also bias the results.

The proposed analogy of blood transfusion leading to specific and nonspecific immunosuppresion in organ transplant recipients and a similar effect in cancer patients undergoing surgery and receiving blood transfusions, is not a sound one. A suppression of the immune apparatus of the host does not necessarily lead to enhanced tumor growth; results ranging from increased to decreased tumor growth have been reported[29-31]. Furthermore, a transplant recipient receives blood transfusions prior to antigenic stimulation of the graft and additionally receives immunosuppressive therapy. In a cancer patient, on the other hand, the assumed tumor antigens have been present for some time prior to administration

of transfusions. Due to heterogeneity of tumor characteristics the results cannot be reciprocated for all tumors, even if blood transfusions result in enhanced growth of some tumors. This could explain the contradictory results reported above.

Irrespective of blood transfusions, surgical trauma in itself has been shown to exert a profound effect on the host's immune apparatus. Inhibition of cell-mediated immunity, decrease in phagocytotic capacity of reticulo-endothelial cells, macrophages and neutrophils; decrease in immunoglobulins and complement have been reported following surgery. Anesthetic agents, which inevitably accompany surgical procedures, have also been implicated in producing immunosuppression in patients[35,36]. Furthermore, other factors often present in cancer patients **i.e.:** poor nutritional state, fever, pharmoceutical therapies in the form of antibiotics, have also been reported to have similar effects on the immune status of the patients[37-39]. Virtually every cancer chemotherapeutic agent is shown to be immunosuppressive [40]. Marked depression of immune-competence has also been reported following blood loss[41,42]. These factors have not been taken into consideration in the reports mentioned above. Thus, several factors other than blood transfusions could explain or play a role in the adverse effect on tumor growth following transfusion in surgically treated patients. Blood transfusion could be an index of either less successful or more difficult surgery or other biological behaviour of malignant tumors, a variable not measured adequately by histopathological staging.

These retrospective studies tentatively show that transfusion is associated with a worse prognosis than that of patients who do not receive transfusions. A more definite answer can only be obtained with experimental studies, since elimination of other factors which may influence the retrospective data, or for that matter a prospective randomized clinical study, is not feasible.

These arguments form the theoretical background to the experiments reported in this thesis.

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CHAPTER 3

ANNOTATIONS

In all studies reported in the following chapters, the central theme of the blood transfusion phenomenon and the modulation of tumor growth is present. Each report is presented as a manuscript valid on its own merit. However, an examination of the studies in the order presented here, may demand clarification of minor technical differences present in the studies. A few explanatory remarks will be made below to provide a perspective for evaluating all experiments centered around a single focus.

3.1. ANIMALS

Male rats of inbred Brown Norway (BN) and WAG strains were used. Serological and cellular tissue typing has shown the WAG/Rij strain to be homozygous for the H-1^{w} haplotype, whereas BN rats are homozygous for the H-1^{n} haplotype. Thus, BN and WAG rij rats are incompatible for class I and class II antigens of the rat's major histocompatibility complex. The animals were used as tumor bearing animals or allogeneic blood donors. They were 16-20 weeks old, and weighed 250-300 grams.

3.2. TUMORS

Tumor LS 175, which arose spontaneously in BN rats and BC 1618, a radiation induced tumor in WAG rat were employed in the studies. Tumor LS 175 is a nonimmunogenic sarcoma and tumor BC 1618 is known to be highly immunogenic in nature. They reflect the array of immunogenic properties generally used in oncological studies.

Tumor LS 175 is maintained in vivo and in vitro. Cultured LS 175 tumor cells were used in the experiments. A possible selection for growth of the tumor cells, is inherent to the use of cells obtained from culture. However, in vitro cells avoid the disadvantages which render in vivo tumor cells inadequate for quantitative and reproducible studies. The greatest disadvantages of employing in vivo tumor cells concern factors capable of influencing the incidence of metastases after intravenous injection of tumor cells **i.e.:**

* difficulty in obtaining a single cell suspension;
* impossibility of obtaining a similar ratio of viable to nonviable cells;
* possibility of the presence of different degrees of host infiltrating cells in the tumor cell suspension.

The effect of experimental procedures on the behaviour of the injected tumor cells was assessed by means of a lung nodule assay **i.e:** Tumor cells were injected i.v. into experimental and control rats. After a certain period of time, animals were sacrificed and the number of colonies developing on the lung surface were counted with the naked eye. The moment of sacrifice of the experimental animals was determined by evaluating the number of colonies present on the lung surface of untreated control rats. An arbitrary number of colonies was considered satisfactory, to avoid extreme numbers of colonies on both side of the scale. This led to the performance of the nodule assay on different days post tumor inoculation in the consecutive studies.

The number of tumor cells injected was determined by means of dose-growth time experiments. The doses used in the studies are based on the aspiration for a practical duration of each experiment of between three and four weeks. Hereby the effect of unknown variables on the outcome of the experiments was kept to a minimum.

3.4. EXPERIMENTAL DESIGN

Knowledge of the precise factors functional in modulating tumor cell behaviour is elementary. In order to avoid confusion by the use of standard descriptions for different aspects of tumor behaviour, a uniform vocabulary usage is recomended. However, the use of certain words in oncological research reports is unfortunate. Two such words are "take" and "growth" of tumor cells; these are also used by us in the following experimental reports. To clarify the situtation, the definition of these words, as employed in this thesis is given below:

Experimental procedures performed before or around the day of tumor injection are considered to affect mainly the "take" of these tumor cells. A period of seven days is assumed to be sufficient for the establishment of the i.v. injected tumor cells. Therefore, the experimental procedures seven days after tumor inoculation are considered to predominantly have an effect on the outgrowth of these established artifical micrometastases. However, the mode of final assessment is to count the number of tumor colonies on the lung surface. The number of nodules counted is assumed to be correlated to the growth of tumor cells. The word "growth" in the following reports is used in this context, and not to denote an increase in size of a tumor lesion.

CHAPTER 4

MODULATION OF TUMOR GROWTH BY ALLOGENEIC BLOOD TRANSFUSION.

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Summary

The effect of a single blood transfusion on the formation and outgrowth of experimental lung metastases was assessed in two tumor models in rats. The transfusions were given either 1 week before (day -7) or 1 week after (day +7) tumor cell inoculation. The first approach was employed to investigate the effect of transfusions on the formation of lung colonies, the second approach to study the effect on the outgrowth of established metastases. The first tumor model used was a transplantable, nonimmunogenic sarcoma(LS 175) in BN rats. Animals were injected i.v. with 10^5 tumor cells and the number of metastases developing in the lungs was counted after 18 days. Experimental animals received 1 ml of allogeneic WAG blood, controls were given 1 ml of syngeneic BN blood. A single allogeneic transfusion given on day -7 had no effect on the formation of LS 175 lung colonies, but when given on day +7, the outgrowth of established metastases was stimulated. The second tumor model was a highly immunogenic transplantable basal cell carcinoma (BC 1618) in inbred WAG rats. Rats were injected i.v. with 10^6 tumor cells and the numbers of lung colonies were counted after 21 days. Experimental animals were transfused with 1 ml of BN blood; controls received 1 ml of WAG blood. Transfusion on day -7 led to a significant inhibition of lung metastases, whereas a transfusion on day +7 had no effect. The results clearly indicate that allogeneic blood transfusions can modulate the growth of tumor metastasis. Although immunological factors seem to play a crucial role in this transfusion phenomenon, there was no clear-cut correlation between the observed effects (accelerated tumor growth vs inhibition of metastases) and the type of immunomodulation evoked.

Introduction

From clinical and experimental studies it is known that allogeneic blood transfusions can have a profound effect on the immune response[1,3]. Blood transfusions given to transplant recipients before grafting have been found to exhibit a beneficial effect on the survival of kidney grafts, presumably by triggering an immunosuppressive mechanism which is however still poorly understood[6,14,18]. In conjunction with immunosuppressive drugs, this effect of blood transfusions is very powerful and has been demonstrated to be a major factor in determining the fate of a kidney graft[13]. Cancer patients undergoing surgery frequently receive blood transfusions which, in addition to the immunosuppressive effects of anesthesia and the operation procedure itself[10,17], may further hamper their immune capacity and influence tumor growth and metastasis. Retrospective studies by Burrows and Tartter[2] performed in patients with colon cancer seem to indicate that blood transfusions may indeed have this effect. It was found that the recurrence free survival rate in transfused patients was significantly lower than in nontransfused patients. In breast cancer patients, a similar trend was observed[19]. In animal studies, detrimental as well as beneficial effects of blood transfusions on tumor growth have been noted[4,11,12]. The present study was undertaken to investigate the blood transfusion phenomenon in two rat tumor models, namely a nonimmunogenic tumor in BN rats and an immunogenic tumor in WAG rats. Both animal strains have been extensively studied before with regard to the effects of blood transfusions on organ graft survival[7,8].

Materials and methods

Animals: Male rats of inbred BN and WAG strains were used. The animals, aged 16-20 weeks, were bred under specific pathogen free conditions. The animals weighed 250-300 grams.

Tumors: Tumor LS 175 is a nonimmunogenic sarcoma which arose spontaneously in the BN rat[9]. The tumor had been transplanted for about 2 years by s.c.serial passage in the BN strain and was subsequently maintained as a stationary culture in Dulbecco's minimum essential medium, supplemented with 10% fetal calf serum(FCS). To obtain cells for the in vivo experiments free floating LS 175 cell clumps were harvested from the tissue culture flasks and, after washing, were resuspended in Hank's balanced salt solution(HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and was between 80% and 90%.

Tumor BC 1618 is a radiation induced basal cell carcinoma which has been transplanted in syngeneic WAG rats for about 5 years. The tumor is highly immunogenic as judged by in vivo immunization challenge experiments carried out according to the method described by Prehn and Main[15]. Tumor cell suspensions

were prepared from s.c. tumor implants in HBSS enriched with 15% FCS. Several sections near the periphery of the tumor were selected and diced into small fragments. Tumor cells were isolated by mechanical disruption using a razor blade mounted on an overhead stirrer as described by Reinhold[16]. Single cells were isolated from nondispersed clumps by sieving through nylon gauze. After two washings in HBSS, the viability was assessed with trypan blue; it was 60%.

Lung Nodule Assay: Tumor cells were inoculated i.v. into experimental and control rats and the number of colonies developing on the lungs were counted after 18 days for tumor LS 175 and after 21 days for tumor BC 1618. The lungs were excised, rinsed in tap water and subsequently fixed in Bouin's solution. Tumor nodules on the lung surface were counted with the naked eye.

Blood Transfusions: BN rats received a single i.v. injection of 1 ml heparinized whole WAG blood; WAG rats were transfused with 1 ml of BN blood. Control animals were injected with 1 ml syngeneic blood or 1ml HBSS.

Experimental Design

Two different protocols were followed. In the first approach, allogeneic and syngeneic transfusions were given 1 week prior to tumor inoculation. This was done to study the effect of transfusions on the formation ("Take") of lung metastases. In the second approach, the transfusions were given 1 week after tumor injection. This was performed to investigate the effect of blood transfusions on the growth of established lung metastases. The significance of the differences between groups was determined by the Wilcoxon rank test and the X^2 test.

Results

Effect of transfusions on "take" of metastases formation:

BN and WAG rats were transfused with 1 ml of allogeneic blood, syngeneic blood, or HBSS 1 week before tumor cell inoculation. The number of lung nodules was counted 18 or 21 days after tumor challenge. The results obtained in BN rats with tumor LS 175 are given in Table 1. Twelve animals were treated with an allogeneic transfusion, 10 received a syngeneic blood transfusion, and 8 rats were given HBSS. Because of the large variation in number of lung metastases within each group, the results were divided into three categories: lungs containing 0-25 nodules, lungs with 26-100 nodules and lungs with more than 100 nodules. As can be seen in Table I, the distribution of metastases was not significantly different in the three experimental groups. The majority of animals (50%-62%) fell into the first category and had between 0 and 25 lung nodules. The results indicate that blood transfusions given on day -7 had no effect on the formation of LS 175

lung colonies. The results obtained with tumor BC 1618 in WAG rats (8 animals per group) were quite different (Table 2). In this model, allogeneic transfusions produced a marked (about 50%) reduction in the number of lung metastases. The difference from the controls which had received a syngeneic transfusion was statistically significant ($P<0.01$).

Table 1: Effect of blood transfusions on the formation of experimental lung metastases of tumor LS 175 in BN rats.

Treatment	Fraction of animals with		
	0-25 nodules	26-100 nodules	> 100 nodules
HBSS	5/8	3/8	0/8
Syngeneic blood	5/10	4/10	1/10
Allogeneic blood	7/12	2/12	3/12

Table 2: Effect of blood transfusion on the formation of experimental lung metastases of tumor BC 1618 in WAG rats.

Transfusion	Mean no. of nodules (± SD)	Range	Median
Syngeneic	131 ± 17.7	98-153	133
Allogeneic	66 ± 9.9	51- 80	66

Effect of transfusions on tumor growth:

One week after tumor cell inoculation, BN and WAG rats were treated with 1 ml of allogeneic blood, syngeneic blood, or HBSS. The lung nodules were counted 11 and 14 days later. Table 3 gives the results obtained in BN rats bearing tumor LS 175 (16-20 animals per group). Animals treated with allogeneic blood had more lung metastases than animals treated with syngeneic blood transfusions or HBSS ($P<0.025$). After a syngeneic transfusion, 69% of the animals had 0-25 metastases and 25% showed 26-100 metastases. However, after an allogeneic blood

transfusion, this distribution was reversed; 19% of the animals fell into the first category and 61% in to the second. The median number of lung colonies in the

Table 3: Effect of ttransfusion on the growth of established lung metastases of tumor LS 175 in BN rats.

Treatment	Fraction of animals with		
	0-25 nodules	26-100 nodules	> 100 nodules
HBSS	10/20	8/20	2/20
Syngeneic blood	11/16	4/16	1/16
Allogeneic blood	3/16	10/16	3/16

Table 4: Effect of blood transfusion on the growth of established lung metastases of tumor BC 1618 in WAG rats.

Transfusion	Mean no. of lung nodules (± SD)	Range	Median
Syngeneic	112 ± 19.0	63-143	121
Allogeneic	101 ± 24.2	72-124	106

allogeneic group was 53, in the syngeneic group 22, and in the HBSS group 23.

Blood transfusions given to WAG rats 1 week after inoculation of tumor BC 1618 (8 animals per group) had no effect on tumor growth. The number of lung metastases observed in the group treated with an allogeneic transfusion did not differ from the number of nodules found in the syngeneic group (Table 4). The mean number of lung nodules was 101 ± 24.2 and 112 ± 19.0 respectively.

Table 5: Summary of the effect of blood transfusion on tumor growth in two tumor models.

Tumor model	Transfusion day − 7	Transfusion day + 7
LS175 in BN rats	No effect	Stimulation
BC1618 in WAG rats	Inhibition	No effect

Discussion

The present data, summarized in Table 5, show that allogeneic blood transfusions can have an effect on the take and growth of experimental lung metastases. In the two models the transfusions used either had an inhibitory effect on the formation of lung metastases (tumor BC 1618) or stimulated the growth of established metastases (tumor LS 175). These diverse results, **i.e.** inhibition of tumor take versus stimulation of tumor growth, mirrors the different effects of blood transfusions on tumor growth as described in the literature. Francis and Shenton[4] observed stimulation of s.c. tumor growth in rats after pretreatment on day -14 with allogeneic blood. In contrast, Oikawa et al.[12] found that pretreatment of WKA rats with allogeneic blood on day -7 led to inhibition of s.c. tumor growth. Interestingly, the latter authors found that the strength of growth inhibition mainly depended on the blood donor strain used. Some rat strains induced a very strong inhibition others only a moderate inhibition, whereas some strains produced no effect at all. In subsequent experiments they further demonstrated that growth inhibition could also be produced by other forms of allogeneic immunization, skin grafting being the most effective[5]. The comparable effects evoked by allogeneic blood transfusions and skin grafting in the above mentioned model suggest that in WKA rats, blood transfusions lead to sensitization and not to immunosuppression as might have been expected. A similar phenomenon occurs in the BN rats used in the present study. From transplantation studies it is known that a single WAG blood transfusion given to BN rats leads to sensitization which is reflected in accelerated rejection of subsequently transplanted organ grafts[7]. In contrast, WAG rats transfused with BN blood exhibit the classical phenomenon of transfusion-induced immunosuppression; the response to mitogens is reduced and organ grafts are accepted for a prolonged period[8]. Consequently, the two models used in the current study could represent the two extremes of the transfusion effect spectrum: **sensitization** in the BN-LS175 model and *immunosuppression* in the WAG-BC 1618 model. Surprisingly, **sensitization** was

associated with **accelerated** tumor growth whereas transfusion-induced *immunosuppression* led to **inhibition** of metastases formation. Francis and Shenton[4] found in their tumor model that allogeneic transfusion led to suppression of T-cell reactivity which was associated with accelerated tumor growth. Nathenson et al.[11] showed that in mice, allogeneic transfusions induced a depressed mixed leukocyte reactivity whereas natural killer(NK) cell activity was increased. In their model, blood transfusions led to a decreased number of pulmonary metastases. These examples, combined with our own diverse results indicate that the enhanced or decreased growth of tumors following blood transfusions is a complicated matter for which several mechanisms, not necessarily all immunological, may be responsible. The mechanisms range from altered NK cell or macrophage activity, increased or decreased T-cell reactivity with corresponding changes in lymphokine production to alterations in humoral immunity. All these factors may influence tumor growth; whether the net result of the immunomodulation will lead to tumor inhibition or tumor promotion may be determined by the immunogenicity of the tumor, sharing of certain epitopes on the tumor with antigens on the immunizing cells, and the immunocompetence of the host.

Current immunological studies with transfused, tumor bearing BN and WAG rats may help to elucidate the blood transfusion phenomenon, and thus enable its manipulation.

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CHAPTER 5

EFFECT OF ANESTHESIA, SURGERY AND BLOOD TRANSFUSION ON TAKE AND METASTATIC GROWTH OF TUMOR CELLS, IN A RAT MODEL.

This chapter was published in Transplant.Proc. 1987; 19:1473-1474 and Surg. Res. Comm. 1987; 2:19-25.

Summary

Several retrospective studies have indicated an adverse effect of blood transfusion on the survival of surgically treated cancer patients. We have investigated the effect of anesthesia, surgery alone and blood transfusions alone and in combination on the growth of artificial lung tumor metastases in BN rats. Furthermore, the effect of blood transfusions and surgery on the take of tumor cells was studied. Abdominal surgery in combination with allogeneic blood transfusion and allogeneic blood transfusion alone was found to have a strong stimulatory effect on the growth of the metastases. Surgery alone and ether anesthesia alone, did not demonstrate this tumor growth enhancing effect. Abdominal surgery on day 0 enhanced the take of tumor cells, while blood transfusions on day -7 had no effect. The immune status and the local environment of the metastases may provide crucial information for understanding this phenomenon

Introduction

The beneficial effect of blood transfusions on the fate of kidney grafts is well recognized[1-3]. Recent retrospective clinical studies seem to indicate a link between blood transfusions and tumor growth in surgically treated patients[4,5]. Blood transfusion induced immunosuppression could be responsible for a detrimental outcome of patients with malignancies, due to promotion of tumor growth. However, anesthesia or surgery itself could be factors contributing to enhanced tumor growth. Anesthesia, the inseparable concomitant of surgical procedures, has also been implicated in the resulting decreased immune competence[10-13]. Surgery, an important tool in the cancer therapeutic arsenal, has been indicated as being immunosuppressive [6,7]. This has been subsequently substantiated by several in vitro studies demonstrating depression of cellular immunity following surgical procedures [8,9]. These immunological modulations following anesthesia, surgery and blood transfusion alone or surgery in combination with the others may also adversely effect tumor growth in cancer patients.

We report here on the effects of anesthesia, abdominal surgery, blood transfusion alone or in combination on artifical metastatic growth, in a rat model. Furthermore,the influence of surgery and blood transfusions on take, homing, of circulating tumor cells is investigated.

Material and methods

Animals: Male rats of the inbred BN and WAG strains were used. The animals, aged 16-20 weeks, were bred under specific pathogen free conditions.

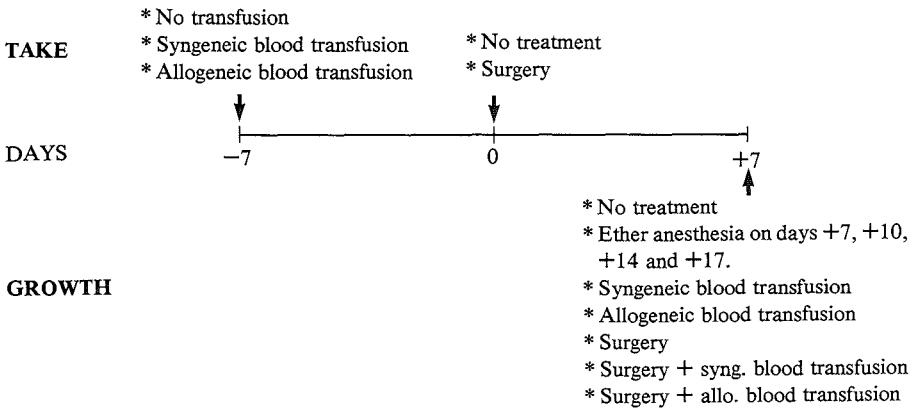
Tumor: Tumor LS 175 is a spontaneous, non-immunogenic sarcoma in BN rats. The tumor is maintained as a stationary culture in Dulbecco's minimum essential medium, supplemented with 10% fetal calf serum (FCS). To obtain cells for the in vivo experiments, free floating LS 175 cell clumps were harvested from the tissue culture flasks and, after washing were resuspended in Hank's balanced salt solution (HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and was between 90 and 95%.

Lung colonies assay: 2.5×10^5 LS 175 tumor cells, suspended in a volume of 1 ml, were injected intravenously into experimental and control rats. The number of colonies developing on the lungs were counted after 24 days. The lungs were excised, rinsed in tap water and subsequently fixed in Bouin's solution. Tumor nodules on the surface of both lungs, visible to the naked eye, were counted.

Blood transfusions: BN rats received a single i.v. injection of 1 ml heparinized whole blood or 1 ml of syngeneic blood. Control animals received no transfusions.

Abdominal surgery: Under ether anesthesia, an ileum resection was performed of a 5-6 cm segment, followed by end to end anastomoses using continuous suturing with 7.0 silk. The procedure lasted 15-20 minutes, with an estimated blood loss of ±2 ml.

Figure 1: Experimental design.



Experimental design

The effect of ether anesthesia on the growth of established metastases was investigated by anesthetizing a group of rats, inoculated with 2.5 x 10^5 tumor cells on days 0, +7, +10, +14, and +17 for a period of 10 minutes (Figure 1). Control animals were injected with tumor cells on day 0 but did not undergo subsequent anesthesia. Each experimental group consisted of 8-12 animals. The effect on growth of artifical lung metastases of abdominal surgery alone, blood transfusion alone or the two in combination with each other, was studied. BN rats were injected with 2.5 x 10^5 tumor cells i.v., on day 0. The control group received no further treatment. On day +7 groups of tumor bearing rats received only syngeneic or allogeneic blood transfusions or additionally underwent abdominal surgery. One group underwent abdominal surgery and received no blood transfusion. The lung nodule assay, read on day +29, was statistically analysed using the Student "t" test, the limit of significance being $P<0.05$.

To study the take (homing) of circulating tumor cells, groups of BN rats received syngeneic or allogeneic blood transfusions on day -7 or underwent abdominal surgery on day 0. On day 0, animals were inoculated with 1 x 10^6 tumor cells. Control animals received tumor cells on day 0 and did not undergo the experimental procedures on days -7 and 0. Survival times of these animals were monitered.

Table 1: Effect of blood transfusions; surgery; and surgery + blood transfusions on "growth" of tumor metastases.

Transfusion on day +7	**No. of animals**	**Mean no. of lung colonies (± S.D.)**	
No. transfusion/surgery	12	13 ± 4	
Syngen. transf.	10	17 ± 6	N.S.
Allogen. transf.	10	26 ± 7	S
Surgery	8	14 ± 8	N.S.
Surgery + syngen. transf.	10	14 ± 4	N.S.
Surgery + allogen. transf.	8	38 ± 10	S

Figure 2: Effect of blood transfusions on tumor "take".

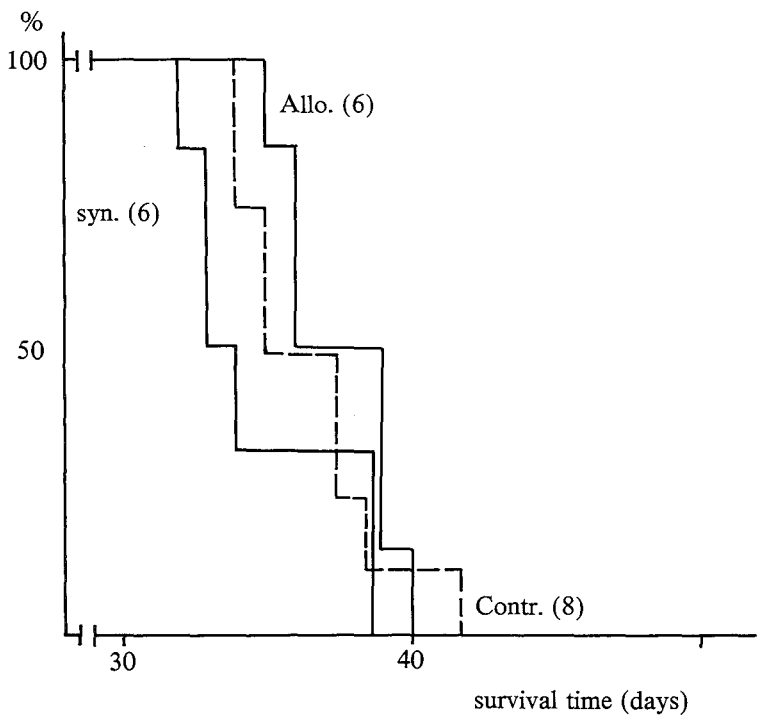
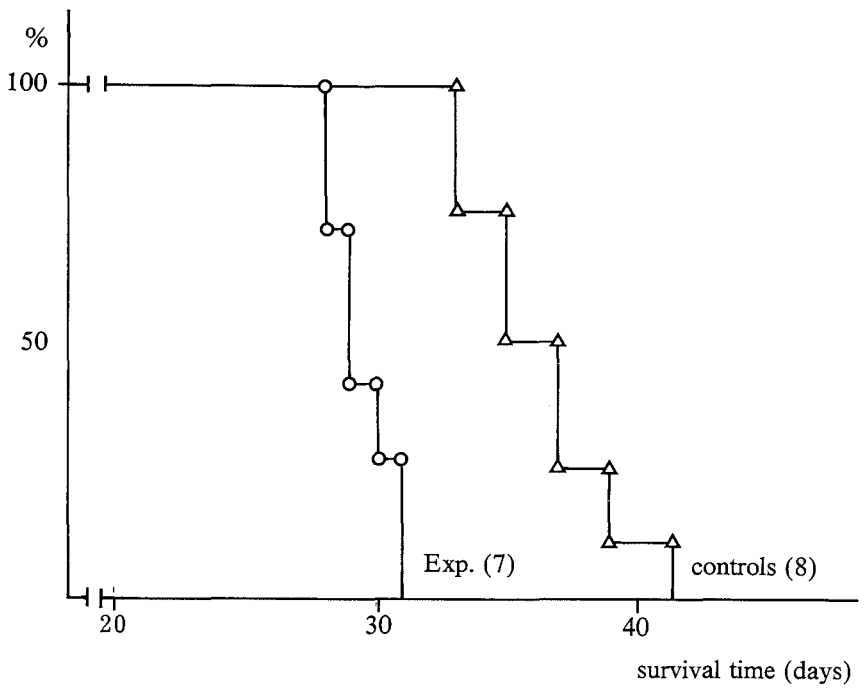


Table 2: Effect of ether anesthesia on "growth" of artifical tumor metastases.

Tumor inoculation on day 0	**No. of animals**	**Mean no. of lung colonies (± S.D.)**	*
No treatment	15	35 ± 22	
Anesthetized on days: + 7; + 10; + 14; + 17	9	22 ± 6	N.S.

N.S.: not significant.

Figure 3: Effect of surgery on tumor "take".



Results

Certain experimental procedures on day +7 had a profound effect on growth of established metastases, created by tumor inoculation on day O (Table 1). The mean number of lung nodules in the control group, on day +29, was 13 ± 4. Abdominal surgery, syngeneic blood transfusions alone and abdominal surgery in conjunction with syngeneic transfusion resulted in 14 ± 8, 17 ± 6 and 14 ± 4 lung nodules, respectively. These numbers are not statistically different from the numbers found in the control group. The mean number of lung nodules in the group which received an allogeneic blood transfusion,was 26 ± 7, which is significantly higher than the number in the control group. Abdominal surgery in conjunction with allogeneic blood transfusion resulted in 38 ± 10 nodules, this also being statistically significantly higher with respect to the control group. Ether anesthesia on days +7; +10; +14 and +17 resulted in 22 ± 6 lung nodules, whilst the mean number of nodules of the control group was 35 ± 22(Table 2). These groups did not differ significantly from each other.

The effect of blood transfusions and surgery on the take is illustrated in Figures 2 and 3. Animals receiving syngeneic or allogeneic transfusion on day -7 showed a similar survival time to the control group(Figure 2). However, the survival time of animals which had undergone surgery on day 0 was significantly shorter than the control group(Figure 3), indicating a strong stimulating effect of surgery on "Take" of the circulating tumor cells.

These results indicate no appreciable effect of ether anesthesia, surgical stress, syngeneic blood transfusion or a combination of the two on growth of artifical tumor metastases. Allogeneic blood transfusion alone or in combination with abdominal surgery elicited a significant enhancement of growth of artificial metastases. Blood transfusion had no effect on the "Take", although surgery resulted in a significantly enhanced homing of the circulating tumor cells.

Discussion

The retrospective data indicating a possible link between blood transfusion and poor prognosis for cancer patients[4,5] is compatible with results reported by Francis et al[14] and Marquet et al[15] in experimental animal models. They are also in accordance with the results in this report, showing that allogeneic blood transfusion leads to enhanced growth of tumor metastases (Table 1). Zeller et al[16] have reported either no effect or an inhibitory influence of blood transfusion, using different mouse strains and tumors. In a different tumor rat combination than the one used here, Jeekel et al[17] and Marquet et al[15] also found no effect of blood transfusion on growth of artifical tumor metastases. These reports strongly suggest that the blood transfusion effect is very much dependent on the properties of the tumor and host. Furthermore, the blood donor-acceptor combination may be an influential factor for the final outcome.

The immunosuppresive effect of surgery is speculated to be of consequence

for therapeutic maneuvers in cancer patient treatment, as it may stimulate tumor growth. In the study presented, neither surgery alone nor surgery in combination with syngeneic transfusion was observed to have any effect on tumor growth (Table 1). However, surgery in combination with allogeneic blood transfusion resulted in significantly enhanced growth of tumor metastases. These results further support the above speculation.

The possibility of an effect of anesthesia on the growth of metastases was addressed by Gaylord et al. in 1916[18]. They suggested an increased frequency of metastases following anesthetization, in a mouse tumor model. A profound effect on the immune apparatus resulting in effects like: transient leucopenia, decreased phagocytosis and mobilization of phagocytic cells, depression of B and T lymphocytes function, has been reported following anesthesia[10-13]. Ether anesthesia is reported to result in an increased number of metastases and a lower 5-year survival rate of breast cancer patients, when compared with halothane narcotized patients[19,20]. The ether anesthesia in our rat-tumor model did not demonstrate an adverse effect. Growth of artificial metastases was similar in control and ether narcotized animals.

In contrast to the effect of allogeneic blood transfusion on growth of established metastases, no stimulating effect of syngeneic or allogeneic transfusion was observed on the take of tumor cells. Surgery was, however, found to enhance the take of the tumor cells. The reason for this difference may be that different factors are of importance in the processes of take and growth of a tumor cell, **e.g.:** NK cells may play a greater role in determining the process of take than growth[21,22]. The present study has demonstrated that allogeneic blood transfusion alone and in combination with surgery causes enhancement of tumor metastases growth. Whereas, surgery alone or combined with syngeneic transfusion, and ether anesthesia alone do not result in a significant acceleration of tumor growth. The take of circulating tumor cells does not seem to be influenced by blood transfusions on day -7; abdominal surgery, on day 0, seems to have a stimulatory effect. In view of previous clinical and experimental reports, the significance of this observed alteration metastases growth and take of tumor cells could be of far reaching importance in clinical practice.

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CHAPTER 6

ENHANCED GROWTH OF ARTIFICIAL TUMOR METASTASES FOLLOWING BLOOD TRANSFUSION:

The effect of erythrocytes, leukocytes and plasma transfusion.

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Summary

Allogeneic blood transfusions have been shown to modulate tumor growth in different ways, depending on the tumor-host models used in the studies. Previously, we have demonstrated the enhanced growth of artificial tumor metastases following allogeneic whole blood transfusion, in a BN rat-LS 175 tumor model. In experiments reported here, we have investigated the effect of transfusion of different blood components on the growth of tumor metastases. Erythrocytes and leukocytes are found to promote the growth of LS 175 tumor metastases in BN rats. A similar effect is observed following whole blood transfusion. Plasma transfusion did not have a effect on tumor growth in this tumor-host model.

Introduction

Clinical and experimental results have shown a prolongation of renal allograft survival, following pre-operative blood transfusions[1-4]. Investigations into which blood components could be responsible for the immuno-suppression after blood transfusions have produced conflicting results. Lymphocytes, erythrocytes and platelets have been shown to be effective by some[5-9], while others were unable to replicate these results [10]. Recent retrospective studies have indicated a link between blood transfusion and shorter disease free interval of cancer patients [11,12]. Employing different host-tumor models, experimental studies have shown reduced, stimulated or no effect on tumor growth following blood transfusions[13,14]. Previously, we have demonstrated enhanced growth of artifical tumor metastases following allogeneic whole blood transfusion, in a rat tumor model[15,16].

In the experiments reported here, we have investigated the competence of different components of blood in modulating metastatic growth. Effect of erythrocytes, leukocytes and plasma transfusion on tumor growth is compared with the effect observed after transfusion of whole blood.

Material and methods

Animals: Male rats of the inbred BN and WAG strains were used. The animals aged 16-20 weeks, were bred under specific pathogen free conditions.

Tumor: Tumor LS 175 is a spontaneous, non-immunogenic sarcoma in BN rats. The tumor is maintained as a stationary culture in Dulbecca's minimum essential medium, supplemented with 10% fetal calf serum (FCS). To obtain cells for the in vivo experiments, free floating LS 175 cell clumps were harvested from the tissue culture flasks and, after washing were resuspended in Hank's balanced salt solution (HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and was between 90 and 95%.

Lung colonies assay: 2.5×10^5 tumor cells, suspended in a volume of 1 ml, were injected intravenously into experimental and control rats. The number of colonies developing on lungs were counted after 24 days. The lungs were excised, rinsed in tap water and subsequently fixed in Bouin's solution. Tumor nodules on the lung surface, visible to the naked eye, were counted.

TRANSFUSIONS:

Whole blood: BN rats received a single i.v. injection of 1 ml heparinized whole blood.

Erythrocytes: Heparinized WAG rat whole blood was centrifuged for 10 minutes at 200 x g, the buffy coat removed and the sediments were pooled and passed in HBSS over a cotton wool column, as described by Diepenhorst et al[17]. BN rats received an i.v. injection of an equivalent number of erythrocytes present in 1 ml whole blood (5×10^9), suspended in a volume of 1ml of HBSS. This suspension was contaminated with <1% leukocytes found in normal blood.

Leukocytes: Whole blood was diluted 1:2 with HBSS. Leukocytes were obtained by centrifuging diluted blood into lymphocyte separation medium (LSM;Bionetics). Residual erythrocytes were eliminated by three successive incubations of the leukocyte enriched suspension, with hemolytic buffer (tris-buffered ammonium chloride 0.17M) and subsequently washed in HBSS. This procedure resulted in marked reduction in the number of erythrocytes (<0.1%). The viability of the remaining leukocytes was determined by the trypan blue exclusion test, and appeared to be >95%. About 1×10^7/ml leukocytes, equivalent to the number of cells in 1 ml of whole blood, were suspended in HBSS and 1 ml of the suspension was administered i.v. to BN rats.

Plasma: Heparinized WAG rat whole blood was centrifuged for 10 minutes at 1500 x g and the resulting supernatant collected. The supernatant was subsequently passed through a filter of 0.45 μm gauze (schleicher & schull; FP030/2). Half a milliliter of the filtered plasma was administered i.v. to BN rats.

Experimental design

Blood constituents responsible for the stimulation of tumor metastatic growth were investigated. BN rats were inoculated with 2.5×10^5 LS 175 tumor cells, on day 0. On day +7 groups of rats received i.v. 1 ml of either whole WAG rat blood or WAG erythrocyte suspension, leukocyte suspension, or 0.5 ml of plasma, respectively. Control animals were not transfused. The animals were sacrificed on day +24 and a lung colony assay was performed. The assay was statistically analysed using Student "t" test, with limit of significance $P<0.05$.

Results

The resulting colonies on the lung surface on day +24 following experimental procedures on day +7, are illustrated in Table 1. The mean number of lung colonies in the group of animals administered whole blood was 68 ± 7. Animals which did not receive any treatment, the control group, had 17 ± 6 lung colonies. Hence, the number of colonies found in the control group is significantly less than animals transfused with whole blood. Groups of animals administered erythrocyte and leukocyte suspension demonstrated mean numbers of colonies of 57 ± 2 and 68 ± 8, respectively. These numbers are also statistically

Table 1: Effect of whole blood, leukocytes and erythrocytes on the growth of established tumor metastases.

Transfusion on day +7	Number of animals	Mean number of lung colonies
Washed leukocytes	9	68 ± 8
Washed erythrocytes	10	57 ± 2
Whole blood	10	68 ± 7
No transfusion	10	17 ± 6

Table 2: Effect of plasma transfusion on growth of established tumor metastases.

Transfusion on day +7	Number of animals	Mean number of lung colonies
Plasma	10	53 ± 11
No transfusion	9	54 ± 12

significantly higher than found in control animals. The numbers found in the experimental groups were similar to one another.

The group of animals receiving plasma transfusion had 53 ± 11 lung colonies(Table 2). The control group of the plasma transfused animals, which did not receive any transfusion, had 54 ± 12 lung colonies **i.e.:** there was no difference between these groups.

Discussion

Different blood components have been shown to be successful in the attainment of effective immunosuppression in organ transplant models[5-8]. Jenkins et al[10] have been able to achieve prolongation of cardiac allografts in rats by lymphocyte transfusates, but were unable to obtain the same effect by an erythrocyte transfusion. Jeekel et al[5] found that a purified donor erythrocyte

transfusion was capable of inducing indefinite survival of rat kidney allografts. Francis et al[13] have demonstrated stimulated tumor growth and depressed lymphocyte reactivity in laboratory animals, following whole blood transfusion. We have shown similarly reduced immunocompetence and metastatic growth[16,18]. The blood donor WAG rat differs for class I and class II antigens, with respect to the BN tumor bearing rats. Erythrocytes in the rat express class I histocompatibility antigens of the A locus at the cell surface but do not express any detectable class II antigenic determinants (B and D locus products). Leukocytes express both class I and class II antigens. Erythrocyte and leukocyte suspensions were equally efficient in stimulating tumor growth as did the whole blood transfusion. This suggests that class I antigens alone or combined with class II antigens is capable of producing the blood transfusion effect observed here. The small degree of leukocyte contamination of the erythrocyte suspension might explain the observed effect of the erythrocyte transfusion. However, Wood et al[19] have also shown suppression of the immune apparatus following transfusion with a highly purified erythrocyte suspension. Kapnick et al[8] have shown that a platelet transfusion, which also lack class II antigens, is also capable of inducing unresponsiveness to skin allografts in mice. In a study of a host-tumor model where allogeneic whole blood transfusion resulted in reduced tumor growth, Oikawa et al.[20] observed a similar effect following administration of red blood cell, leukocyte and platelet transfusions. This observation and our results here suggest that different blood components are capable of producing similar results to those obtained following whole blood i.v. transfusion, in a particular host-tumor model. In analyses of the retrospective data of surgically treated cancer patients, Blumberg et al.[12] found a relationship between the greater amount of blood and blood component transfused and a higher incidence of recurrence and death. One explanation could be the presence in plasma of a substance capable of reducing immune function in the transfused patients. Margolese et al.[21] have demonstrated a poor response of breast cancer patients' lymphocytes to T cell growth factor, in the presence of viruses. Furthermore, growth of transplanted tumors in mice is shown to be greatly enhanced following plasma transfusion[22]. Francis et al.[13] have also reported an increase in plasma suppressive activity after allogeneic transfusion and not following syngeneic or saline infusion in rats. However, our results do not support the hypothesis of the presence of a substance capable of modulating tumor growth.

To summarize, erythrocyte and leukocyte transfusions are capable of producing enhanced growth of tumor metastases, the effect being similar to that of whole blood transfusion. Plasma transfusion has no effect on tumor growth. Class II antigens do not appear to be a crucial factor in precipitating the blood transfusion effect, since the same effect was observed after transfusion of erythrocytes bearing only Class I antigens.

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CHAPTER 7

EFFECT OF SURGERY AND BLOOD TRANSFUSION ON IMMUNE PARAMETERS IN A RAT MODEL.

NATURAL KILLER CELL ACTIVITY; GAMMA INTERFERON PRODUCTION CAPACITY; MITOGEN STIMULATION; LEUKOCYTE PHAGOCYTIC ACTIVITY.

This chapter has been submitted for publication.

Summary

Allogeneic blood transfusion alone and in combination with abdominal surgery was found to have a strong positive effect on the growth of the tumor metastases. Surgery alone did not demonstrate this effect(chapter 5). Following blood transfusions and surgery alone and in combination, immune parameters like Natural killer cell activity; Gamma Interferon Production Capacity; T-lymphocyte mitogen blastogenic stimulation; and circulating leukocyte phagocytic capacity were monitored. A correlation between decreased mitogen blastogenic stimulation and increased tumor growth is observed, indicating a role of T-lymphocytes in this blood transfusion phenomenon. This is indirectly supported by the increased tumor growth observed following cyclosporin treatment. Other immune parameters did not show a correlation with modified tumor growth. The immune status of the local environment of the metastases may provide crucial information for understanding this phenomenon.

Introduction

The concept that pretransplant blood transfusion improves the outcome of kidney grafts is now widely accepted [1-3], although there is no consensus regarding the mechanism for this phenomenon. A suppressed immune-response is generally considered to be the path leading to better graft survival. Francis et al and our group have demonstrated that allogeneic blood transfusion has an enhancing effect on tumor growth in certain animal models[4,5]. A number of in vitro studies have confirmed the presence of post-transfusion immunosuppression [6,7]. Surgery, an important tool in the cancer therapeutic arsenal, has been indicated as being immunosuppressive [8,9]. This has since been substantiated by several in vitro studies demonstrating depression of cellular immunity following surgical procedures[10,11]. Immunological modulation following surgery in combination with blood transfusion may be a contributing factor in adversely affecting tumor growth in cancer patients.

Cyclosporin A(Cy A) is a fungal metabolite and the first of a new generation of immunosuppressive agents with a specific site of action within the immune system. The mechanism of action of Cy A is not fully understood but it appears to affect T-cells at an early stage in their transformation[12,13]. Gordon and Singer have suggested that a sub-population of T-cells is particularly susceptible to the drug[14]. Eccles et al. have demonstrated a greatly increased metastasis of lymphomas and sarcomas in a rodents-tumor model, following Cy A treatment[15]. Cyclosporin A offers the possibility of studing the modulation of tumor growth in syngeneic hosts, subjected to specific immune suppression.

Here we report the results of in vitro tests performed to quantitate the immuno-modulating effects following the experimental procedures: surgery alone, blood transfusion alone or the two procedures in conjunction. The immune status of the animals was determined by the following parameters: Natural Killer cell (NK) activity; Gamma- Interferon Production Capacity (GIPCA); leukocyte phagocytic capacity and PHA and Con A mitogen blastogenic response. Furthermore, the drug cyclosporin is used to investigate the validity of the proposed correlation between tumor growth and T-cell function.

Material and methods

Animals: Male rats of the inbred BN and WAG strains were used. The animals, aged 16-20 weeks, were bred under specific pathogen free conditions.

Tumor: Tumor LS 175 is a spontaneous, non-immunogenic sarcoma in BN rats. The tumor is maintained as a stationary culture in Dulbecco's minimum essential medium, supplemented with 10% fetal calf serum (FCS). To obtain cells for the in vivo experiments free floating LS 175 cell clumps were harvested from the tissue culture flasks and, after washing were resuspended in Hank's balanced salt solution (HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and was between 90 and

95%.

Blood transfusions: BN rats received a single i.v. injection of 1 ml heparinized whole allogeneic blood or 1 ml of syngeneic blood. Control animals received no transfusions.

Adominal surgery: Under ether anesthesia, an ileum resection of a 5-6 cm segment was performed followed by end-to-end anastomoses using continuous suturing with 7.0 silk. The procedure lasted 15-20 minutes, with an estimated blood loss of ± 2 ml.

Cyclosporine : Cyclosporin dissolved in olive oil was administered in a dose of 5mg/Kg i.m..

Lung colonies assay: 2.5 X 10^5 tumor cells, suspended in a volume of 1 ml, were injected intravenously into experimental and control rats. The number of colonies developing on lungs were counted after 24 days. The lungs were excised, rinsed in tap water and subsequently fixed in Bouin's solution. Tumor nodules on the lung surface, visible to the naked eye, were counted.

NK Cell assay:

Lymphocyte cell preparation:

The spleens from rats were cut into fragments and pressed through a 100 m sieve. Lymphocytes were isolated using a Ficoll Isopaque gradient, washed and resuspended with 5 ml RPMI containing 10% FCS and incubated for 1 hour at 37°C in tissue culture flasks. Thereafter, the cell suspension was adjusted to a concentration of 20 x 10^6 living cells per ml.

Chromium (^{51}Cr) release assay for NK cell activity:

Natural cytotoxicity was measured in a 4-hr assay using ^{51}Cr labeled YAC target cells [16,17]. Two million target cells were incubated with 400μCi of Na_2 $^{51}CrO_4$ solution (specific activity 50-400μCi/mg ^{51}Cr; Amersham, U.K.) for 1 hr at 37°C. The ^{51}Cr labeled targets were washed twice, counted and resuspended at a concentration of <0.1 x 10^6 per ml. All assays were performed in triplicate in round-bottomed microtiter plates (Nunc, Denmark) in a total volume of 0.2 ml of RPMI 1640 supplemented with 10% FCS. In all assays, 1 x 10^4 targets were added to each well. Lymphocyte effector cells were added at various concentrations according to the chosen lymphocyte-to-target cell ratio 25:1, 50:1, 100:1 and 200:1 respectively. The microtiter plates were centrifuged for 3 minutes at 150 x g and then incubated for 4 hr at 37°C in a humidified 5% CO_2 incubator. To harvest the assay, plates were centrifuged for 1 minute at 150xg and the supernatants were removed using the method described by Hirschberg et al[18]. The release of ^{51}Cr was determined by counting radioactivity in a gamma counter (LKB Wallace Ultragamma II (280).

Specific cytotoxicity:

The percentage specific lysis in all experiments was calculated according to the formula:

$$\% \text{ Specific lysis} = \frac{\text{mean exp. release - mean spontaneous release}}{\text{mean max. release - mean spontaneous release}} \times 100$$

The maximum release was calculated by adding 10% cetavlon (ICI UK) to an aliquot of target cells. Spontaneous release was defined as the ^{51}Cr released from target cells incubated with medium alone. This value was usually 6-10% of the maximum. Mean counts and standard deviation were determined in triplicate tests.

Max % specific lysis was obtained with a lymphocyte-to-target cell ratio of 100:1 and thus only these results are reported here. The percentage specific lysis of the control group is taken as 100% and other results are expressed as % relative to the control group.

Concanavalin A induced gamma interferon production capacity (GIPCA):

Lymphocytes were suspended at $1x10^6$ cells per ml RPMI 1640 containing 10% FCS, 50 I.U. penicillin, 50 μg streptomycin, 2 mM L-glutamine and 10^{-5}M 2-mercaptoethanol. 1 ml of this cell suspension was supplemented with 7.5 g Con A and incubated for 3 days at 37°C in a 5% Co_2 humidified atmosphere. The supernatant from each culture was then harvested and the anti-viral activity determined by the inhibition of the cytopathogenic reduction assay by a dye uptake method using Ratec cells and 100 TCID 50 vesicular stomatitis virus as challenge. GIPCA of the control group is taken as 100% and GIPCA of the experimental groups are expressed as % relative to the value of the control group.

PHA and CON A stimulation assay:

Lymphocyte concentration was adjusted to 7.5 x 10^6 cells per ml in RPMI 1640 medium containing 10% FCS. Cultures of 200 l containing 1μg PHA-P (Wellcome, UK) and 1μg Con A respectively were maintained for 3 days at 37°C in a 5% CO_2 humidified incubator. Six hrs prior to termination, each culture was labeled with 0.8μCi of methyl-3-H-thymidine (^{3}H-Tdr, specific activity 2Ci/mmol; Amersham, UK). After a period of 6 hrs, cultures were harvested with an automatic harvester (microtiter-automash, Dynatech, Holland). Cells were collected on fiber glass filters and after drying, the filters were placed in scintillation vials, 3 ml scintillation fluid added and uptake of ^{3}H-Tdr determined with a liquid scintillation counter (B, searl isocap II; efficiency 96%). ^{3}H-Tdr uptake by the control group was equated to 100 % and the values of other groups were expressed as relative response to the control value.

Phagocyte activity of leukocytes:

Peripheral blood was obtained by cardiac puncture and 2 ml of blood was mixed with an equal volume of Plasmasteril(R) (Fresenius, Bad Homberg, FGR). The mixture was incubated at 37^0C for one hr after which the leukocyte-rich supernatant was collected and washed twice in HBSS. The final leukocyte suspension was adjusted at 5 x 10^6 leukocytes/ml. The phagocyte assay was performed in triplicate in 12.5 ml round-bottomed glass tubes by mixing 0.05 ml of the leukocyte suspension with 0.05 ml of a suspension containing $5x10^8$/ml carboxylated microspheres of diameter 1.74 (Polysciences Inc.,Warrington, PA, USA). The tubes were incubated for 1 hr at 37°C in a gently shaking water bath. The leukocytes were then stained with cristal violet dye after which the degree of phagocytosis was determined in a hemocytometer chamber. By counting two hundred leukocytes, the percentage of phagocytic cells and the average number of ingested microspheres per phagocytic cell was calculated. By multiplying these two numbers a phagocytic index (=number of ingested microspheres per 100 leukocytes) was obtained. The index was used to determine whether there was a significant difference between experimental and control values.

Experimental design

A: An assessment was carried out of immuno-parameters of the control and experimental groups. Following tumor inoculation on day O, animals received syngeneic or allogeneic blood transfusion, underwent abdominal surgery alone or in conjunction with blood transfusion, on day +7. Immuno-parameters were determined on days +8, +11 and +15. Parameters assessed were NK activity; -interferon production capacity (GIPCA); leukocyte phagocytic capacity and; PHA and Con A mitogen stimulation response capacity. Each group comprised 5 animals. Naive animals, n=5, were taken as a control group for each experimental group. The statistical significance was determined by the Student "t" test with limit of significance $p<0.05$.

Immune-parameters of naive BN rats were compared with parameters of BN rats bearing tumor metastases, over the period day 0 to +15 post-tumor inoculation. No differences in the values were found (data not shown) and thus parameters of naive rats were taken as control values in subsequent result analyses. The results of the different experimental groups were made comparable by expressing them in terms of relative responses index, taking the values of naive control BN rats as 100%.

B: On day 0 BN rats were inoculated with $2.5x10^5$ LS 175 tumor cells. Control animals received no further treatment. On days +7, +10 and +14, the experimental animals received cyclosporine in a dose of 5mg/Kg i.m. All animals were sacrificed on day +28 and sebsequently a lung colonies assay was performed. The results were statistically tested by the Student "t" test with limit of significance $p<0.05$.

Figure 1: Effect of blood transfusion and surgery on NK-cell activity.

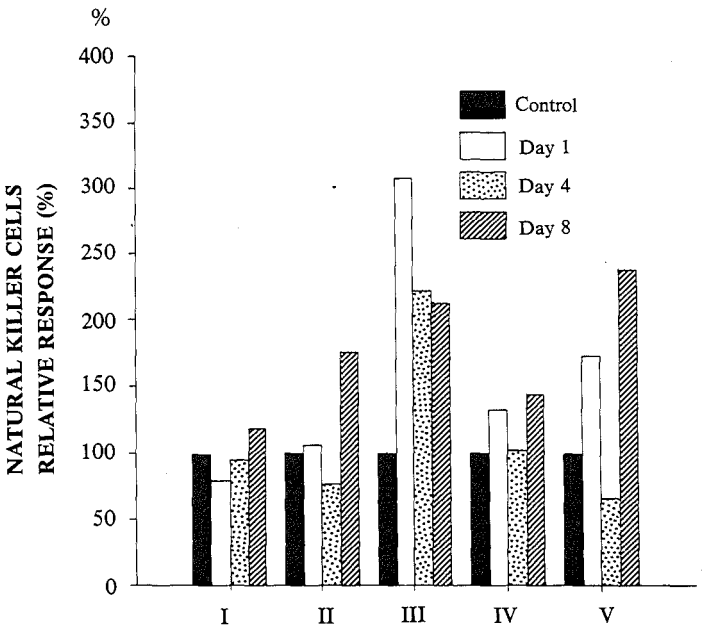


Figure 2: Effect of blood transfusion and surgery on Con-A T-cell stimulation test.

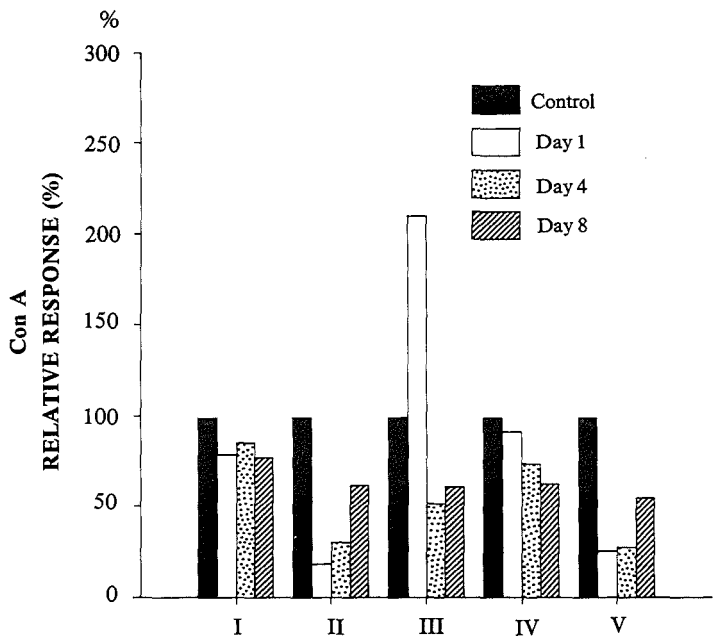


Table 1: Immune parameters expressed as relative response index.

	Days	N.K.	Con A	PHA	Gipca	LPC
Syngeneic transfusion	+ 8	80	(**79**)	nd	74	N
	+ 11	96	86	69	65	N
	+ 15	119	(**78**)	(**58**)	47	N
Allogeneic transfusion	+ 8	106	(**19**)	70	(**164**)	(↓)
	+ 11	76	(**31**)	(**64**)	(**210**)	N
	+ 15	(**176**)	(**53**)	(**194**)	122	(↑)
Abdominal surgery	+ 8	(**309**)	(**211**)	87	nd	(↑)
	+ 11	(**223**)	(**52**)	85	nd	(↑)
	+ 15	(**214**)	(**62**)	93	nd	(↑)
Abdominal surgery +	+ 8	133	93		68	(↑)
syngeneic transfusion	+ 11	102	(**74**)	47	46	(↑)
	+ 15	(**145**)	(**63**)	(**27**)	50	(↑)
Abdominal surgery +	+ 8	(**173**)	(**26**)	90	102	(↑)
allogeneic transfusion	+ 11	66	(**29**)	(**48**)	130	(↑)
	+ 15	(**238**)	(**56**)	334	163	(↑)

(**X**): statistically significant from control group.
nd: not done.

Key to figures 1-4:
Group I : Syngeneic transfusion on day +7.
Group II : Allogeneic transfusion on day +7.
Group III : Abdominal surgery on day +7.
Group IV : Abdominal surgery + syngeneic transfusion on day +7.
Group V : Abdominal surgery + allogeneic transfusion on day +7.

Results

A: Immune parameters following abdominal surgery and blood tramsfusion

Immune parameters were determined to evaluate the effect of blood transfusion, abdominal surgery and a combination of the two procedures on the immune system in artificial micrometastases-bearing BN rats. BN rats were inoculated with tumor cells on day 0. On day +7 animals received syngeneic or

Figure 3: Effect of blood transfusion and surgery on PHA T-cell stimulation test.

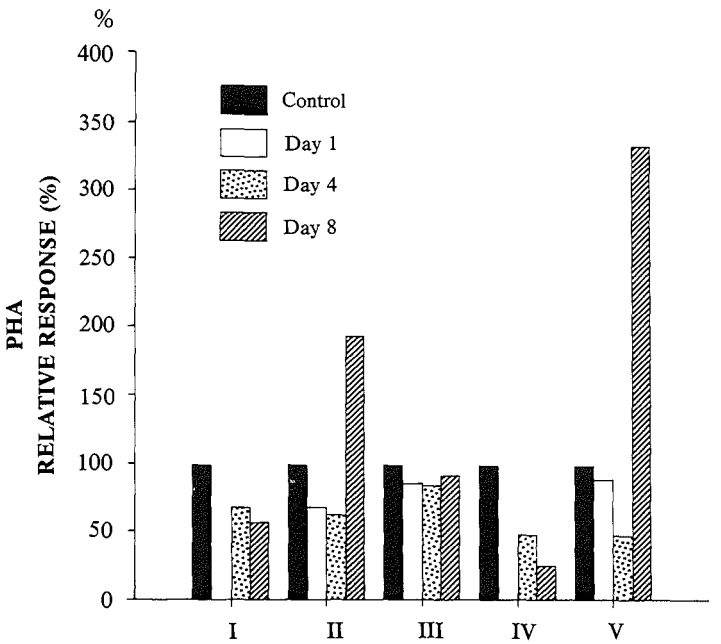
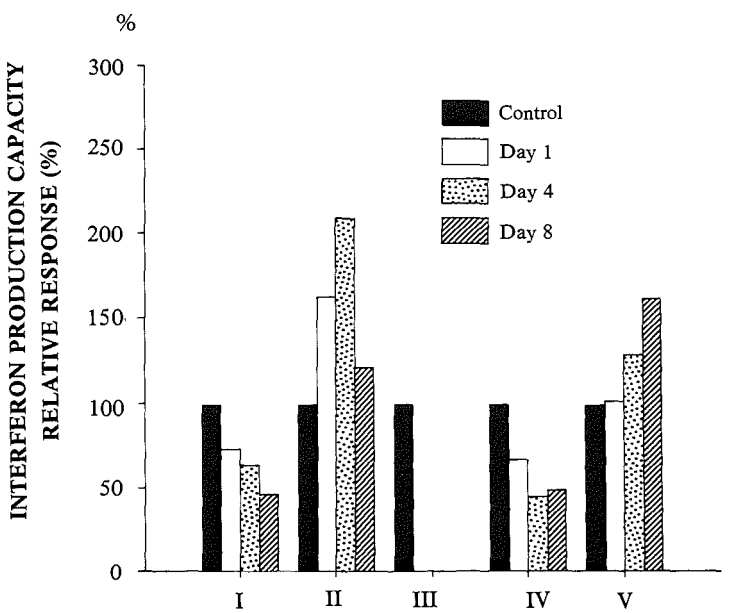


Figure 4: Effcet of blood transfusion and surgery on gamma-interferon production capacity assay (GIPCA).



allogeneic blood transfusion alone or additionally underwent abdominal surgery. One group underwent abdominal surgery and received no transfusion. Immuneparameters on days +8, +11 and +15 were subsequently determined.

NK-cell activity:

Syngeneic blood (group 1) transfusion had no effect on NK cell activity (Table 1, Figure 1). A significant increase of NK cell activity was observed, 8 days after administration of allogeneic blood (group II). Abdominal surgery (group III) resulted in a marked increase of NK cell response one day after surgery and remained significantly above control values for a period of 8 days. Surgery in conjuction with syngeneic blood transfusion (group IV) also resulted in an elevation of NK response, but this occured at a much later stage, **i.e.:** 8 days post-surgery. Surgery in conjunction with allogeneic transfusion (group V) showed a significantly higher NK cell response on the 1st day following the experimental procedure: this was normal by the 4th day and was significantly higher again on the 8th day. The results indicate an increase in NK cell activity following allogeneic transfusion and abdominal surgery. Surgery in conjunction with allogeneic blood transfusion also tends to show a higher response with a transitional drop at 4 days.

Mitogen response:

The mitogen response was significantly reduced or normal, in all experimental groups (Table 1, Figure 2 and 3). The response to Con A and PHA of syngeneic transfused rats was significantly lower on the 1st day after transfusion, but returned to normal by the 4th day and dropped again on the 8th day. Allogeneically transfused rats showed a significantly lower response to Con A throughout the follow up period. In these rats, the response to PHA on the 1st day after transfusion was normal but became significantly reduced on the 4th day and then significantly higher than normal on the 8th day following transfusion. Abdominal surgery alone and in conjunction with syngeneic or allogeneic transfusion led to a normal response to PHA stimulation, with only a transitional decrease in responses on the 4th day following allogeneic transfusion and the 8th day after syngeneic transfusion. However, one day after abdominal surgery a significantly stronger response to Con A (relative response index of 211) was observed with subsequent significantly lower responses 4 and 8 days after surgery. Surgery in combination with syngeneic and allogeneic blood transfusion demonstrated a significantly lower response to Con A, throughout the observation period. Furthermore, the response in the group surgery + allogeneic transfusion overall was lower than the group surgery + syngeneic transfusion (Table 1).

GIPCA:

The gamma-interferon production capacity (GIPCA) of animals administered syngeneic transfusion alone or in conjunction with surgery was comparable to the GIPCA of control rats(Table 1, Figure 4). Allogeneic blood transfused rats displayed a significantly stronger GIPCA on the 1st and 4th days after transfusion, with relative response indices of 164 and 210, respectively. On the 8th day after transfusion, the GIPCA response returned to normal. The group receiving syngeneic or allogeneic transfusions in addition to abdominal surgery showed GIPCA values similar to the control group. Thus, except for a slight increase in GIPCA following allogeneic transfusion, no other experimental procedure affected this parameter.

Phagocytosis:

Following allogeneic transfusion, a significant drop in leukocyte phagocytic capacity was observed. It returned to normal on the 4th day after transfusion and then became significantly higher by the 8th day (Table 1). Following abdominal surgery, alone or in combination with syngeneic or allogeneic transfusion, a significantly elevated phagocytic capacity was encountered throughout the follow up period.

Table 2: Effect of cyclosporine A on growth of established metastases.

Tumor inoculation on day 0	**No. of animals**	**Mean no. of lung colonies (± S.D.)**
No treatment	10	17 ± 6
Cyclosporine-A (5 mg/kg/doses, i.m.) on days: + 7; + 10; + 14	9	60 ± 12*

*: Statistically significant.

B: The effect of cyclosporine on tumor growth.

The results of the experimental animals are illustrated in Table 2. In control animals, 17 ± 6 lung colonies were found on the lung surface, whereas the Cy A treated group had 60 ± 12 colonies. The number of colonies in the Cy A treated

group was significantly higher than in the control group, $P<0.001$ according to the Student "t" test.

Discussion

NK cells are believed to have an important role in immune surveillance and inhibition of growth of tumors. High NK cell activity in vitro has been reported to correlate with resistance to tumor growth in experimental models [19,20]. Furthermore, a low NK cell response has been reported in lymphoma patients[21]. In the present study, we found no such correlation between NK cell response and metastatic growth, as reported in chapter 5. Fodstad et al[22] have reported a similar lack of correlation in mouse experiments. This may indicate a relatively minor, if any, role of NK cells in determining the growth of established artifical metastases, in our model.

Reticulo-endothelial(R.E.) phagocytic activity has been reported to be profoundly diminished following surgical stress[23]. We measured the phagocytic capacity of the circulating leukocytes, which play an important role in the overall defence mechanisms of an organism[24,25]. However, in contrast to the effect on the phagocytosis capacity of R.E., a significant increase was observed in the phagocytic activity of circulating leukocytes following surgery alone or in combination with blood transfusion. Similar results are reported in the post-operative period in patients undergoing surgery[26]. No exact mechanism for these observations is known. The mitogen blastogenic response has been reported to be diminished following blood transfusion[6,7] and surgery[27]. Francis et al[5] have shown a significant decrease in lymphocyte reactivity and increased tumor growth in animals which had received an allogeneic blood transfusion. The results given in Table 1 demonstrate a statistically significant depression of blastogenic response following blood transfusion, surgery alone or surgery in combination with blood transfusion. Furthermore, a greater decrease in response is observed in animals administered allogeneic transfusion alone or allogeneic transfusion in conjunction with surgery. This is compatable with the observed increase in the growth of tumor metastases (chapter 5). The number of colonies in the Cy A-treated group was significantly higher than in the control group. These results further substantiate a possible role of T-cells in the modulation of tumor growth.

Although the downward trend of response is similar for Con A and PHA stimulation, the results (Table 1) show some differences between the responses of the two mitogens. A possible explanation for these differences could be the fact that Con A stimulates T-lymphocytes, while PHA stimulates predominantly T-lymphocytes but also B-lymphocytes to a certain extent.

There is yet no concordance about the role of interferon in the defence against tumors. Interferon has been reported to be capable of increasing the lethal effect on tumor cells, by NK cell and macrophage stimulation[28]; therefore, following the experimental procedures, GIPCA was performed. No correlation was found between GIPCA and the observed growth of tumor

metastases after the experimental procedures. A similar lack of correlation has been reported by Moller-Larsen et al[29]. Furthermore, the GIPCA test employed here is more sensitive to measure an increase rather than a depression in interferon production. This could be an explanation for the discrepancy in the depressed mitogen stimulation response and the absence of changes in GIPCA values.

To summarize, only mitogen blastogenic responses seemed to be important parameters with respect to the enhanced tumor growth by blood transfusions, indicating a predominant role for T-cells in this phenomenon. However, all parameters were determined for peripheral blood and spleen lymphocytes, factors present locally in the area of the metastases may be of paramount importance. There is a great need for further investigation of the local factors and elucidation of the mechanism underlying the observed effects.

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CHAPTER 8

ARE GROWTH FACTORS INVOLVED IN THE BLOOD TRANSFUSION PHENOMENON?

Summary

Allogeneic blood transfusions are shown to be capable of modifying tumor growth. We have previously demonstrated a correlation between modified tumor growth and the T-cell function of transfused animals. Recently, several cytokines have been shown to affect the function of many different immune competent cells. We hypothesized that growth factor(s) might be induced by allogeneic transfusion. The validity of this suggestion was tested by assaying the growth of tumor cells in vitro. The results of this experiment do not support the speculation of an important role of growth factors in the blood transfusion phenomenon.

Introduction

The beneficial effect of pre-transplant blood transfusion on organ transplant survival is a generally accepted fact. However, the underlying mechanism has remained elusive. Several hypotheses **e.g.:** clonal deletion, anti-idiotypic and suppressor T-cells, have found supportive evidence from clinical and experimental studies[1-3]. All these mechanisms envisage a predominant role of T-cell function. The effect of blood transfusion on tumor growth has been reported in chapters 4 and 5. In this blood transfusion effect, a correlation with T-cell function has also been demonstrated [chapter 7].

In recent years, increasing numbers of cytokines have been isolated. Cytokines are thought to be the mode of communication between different cells and have been shown to affect the functioning of different cells in vivo. Murine virus-transformed sarcoma cells have been shown to secrete transforming growth factors, which strongly stimulate soft agar colony formation[4]. Activated mononuclear cells produce Interleukin I (IL_1) which is capable of fibroblast proliferation[5]. Platelet-derived growth factors and platelet-derived angio-genesis factor are found to be potent mitogen- and chemoattractants[6,7]. Macrophages from ovarian, breast, colon, or lung adeno-carcinomas have been demonstrated to secrete soluble factor(s), capable of enhancing colony formation of human tumor cell lines[8]. Many more cytokines with a variety of cell function modulating properties have been isolated.

We hypothesized that allogeneic blood transfusion could form a trigger for the release or production of systemic factor(s), which directly or indirectly modify the functioning of immunological and tumor cells. This then could result in an immunosuppresive state leading to better organ transplantion survival or, in cancer patients to enhanced tumor growth. In this communication we report the results of an in vitro study to verify this hypothesis.

Material and methods

Aninals: Male rats of the inbred BN and WAG strains were used. The animals, aged 16-20 weeks, were bred under specific pathogen free conditions.

Tumor: Tumor LS 175 is a spontaneous, non-immunogenic sarcoma in BN rats. The tumor is maintained as a stationary culture in Dulbecco's minimum essential medium, supplemented with 10% fetal calf serum (FCS). To obtain cells for the in vivo experiments, free floating LS 175 cell clumps were harvested from the tissue culture flasks and, after washing, were resuspended in Hank's balanced salt solution (HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and was between 90 and 95%.

Bood transfusions: BN rats received a single i.v. injection of 1 ml heparinized whole WAG blood or 1 ml of syngeneic blood. Control animals received no

Figure 1: Growth rate of tumor cells in serum obtained one day after blood transfusion.

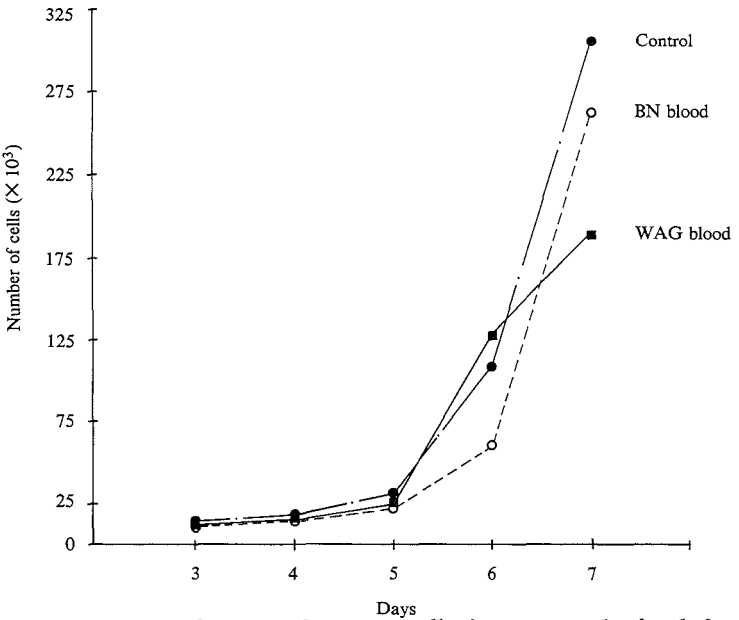


Figure 2: Growth rate of tumor cells in serum obtained four days after blood transfusion.

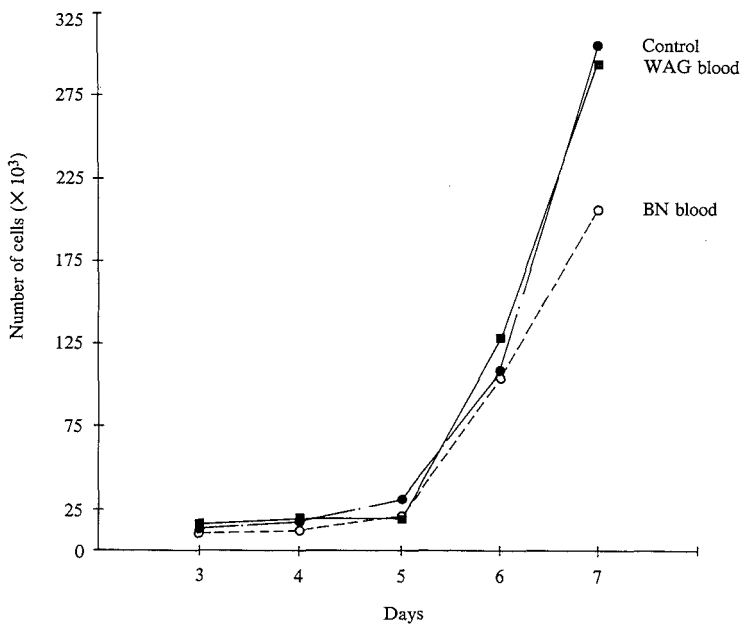
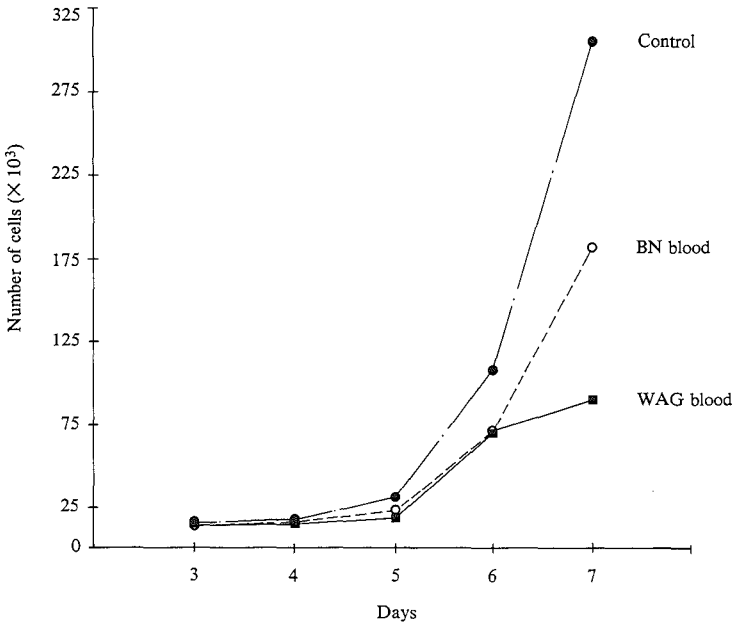


Figure 3: Growth rate of tumor cells in serum obtained eight days after blood transfusion.



transfusions.

Tumor cell growth assay: LS 175 tumor cells were suspended at $5X10^3$ cells per ml in RPMI 1640, containing 5% rat serum (from normal or experimental animals), 1% penicillin and streptomycin and 1% L-glutamine. Two ml of this cell suspension was incubated in flat bottomed titer plates at 37^0C in a 5% CO_2 humidified incubator. On days 3 to 7 after incubation, the mean number of tumor cells per well was determined in triplicate.

Experimental protocol

Growth rate of tumor cells in the serum of transfused BN rats was assessed and compared to that in normal BN rat serum. A group of animals received allogeneic or syngeneic blood transfusion 7 days after tumor cell inoculation. On days +8, +11 and +15, animals were sacrificed and serum obtained from the peripheral blood. Tumor cell growth rate assay was performed, utilizing serum from the different experimental groups. Normal BN rat serum was used as control. The statistical significance was assessed by Student "t" test, with limit

Results

The proliferation rates of tumor cells incubated with serum of blood transfused rats is illustrated in Figures 1-3. The growth rate of tumor cells incubated in serum of rats transfused one day previously is similar to the cells incubated with serum of normal BN rats. Allogeneically or syngeneically transfused rat serum did not show any difference in the effect on tumor cells(Figure 1). No difference was found in growth rate of cells incubated in the serum of BN rats transfused 4 or 8 days previously(Figure 2 and 3). Statistically significant differences were never observed.

Discussion

Enhanced tumor growth is observed following immunosuppresive procedures **e.g.:** multiple operations[9]. Constantian et al have demonstrated that the immunosuppresive effect of operative and accidental trauma is correlated with the presence of a polypeptide in the serum of these patients[10]. Peripheral blood lymphocytes of patients with advanced breast cancer are shown to be inhibited in their response to mitogenic stimuli. This suppressive effect on lymphocytes is abrogated by addition of T-cell growth factor (TCGF) to these lymphocytes in culture[11]. In our experiments, no difference in growth rate was found between tumor cells incubated in normal rat serum or serum from syngeneically or allogeneically transfused rats. This seems to indicate a lack of presence of extra growth factors in the serum of transfused rats. Furthermore, preliminary results of a study of the effect of a known platelet growth factor, in the same experimental settings as used above, also indicate no effect on the tumor growth rate. These results would seem to indicate that growth factors are not important for the rate of tumor cell growth. However, the assay was performed in serum obtained from peripheral blood, whereas cytokines are shown to be optimally active in the immediate environments of the tumor cells and are known to remain active for only a very short period of time. Kanayama et al have found that the serum from splenic venous blood of advanced gastric cancer patients possesses greater immuno-suppressive activity, compared to serum from peripheral blood[12]. This indicates a site dependent activity measurable in serum. It could be an important factor, contributing to our failure to detect any growth modifying capability of serum from transfused animals. Furthermore, the first serum sample was obtained one day after administration of blood transfusion, and it is possible that the changes in cytokines, sufficiently great to be detectable in serum, took place at an earlier stage. Recently, evidence has been presented[13,14] indicating secretion of autocrine growth factor(s) by malignant cells. Blood transfusion may have stimulated the production of autocrine growth factor, with these factors being effective only in the direct vicinity of the cells. This could be yet another explanation for our failure to detect such factors in an in vitro assay. Although we were unable to observe effects to support our hypothesis regarding the induction of growth factor, other assays which take the

above mentioned importance of local environment into account may give a more meaningful answer regarding the validity of the hypothesis.

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CHAPTER 9

CONSEQUENCES OF BLOOD LOSS ON GROWTH OF ARTIFICIAL METASTASES.

This chapter will be published in The British J. of Surgery.

Summary

Previous studies have shown that the rate of growth of lung metastases from a non-immunogenic sarcoma (LS 175) in rats was greater in blood transfused rats. Enhanced growth was also observed after abdominal surgery combined with allogeneic blood transfusions. Syngeneic blood transfusions had no effect. These experimental findings have been confirmed in retrospective clinical studies. The allogeneic blood tranfusion effect may be omitted in cancer patients by means of autologous blood transfusions. For such a transfusion policy, blood donation prior to surgery is needed. The aim of the present study was to investigate the effect of blood loss prior to surgery on formation ("Take") of lung colonies, and on the outgrowth of established metastases in the BN rat model. These aspects of tumor behaviour were also investigated in rats undergoing surgery and/or receiving blood transfusion after blood loss. The results indicate that blood loss has a profound stimulating effect on the growth of established metastases, but not on the "Take" of tumor cells. This stimulating effect was also found to be present when blood loss was combined with surgery, while previously, surgery alone was found to have no effect. Both allogeneic and syngeneic transfusions in combination with blood loss had a strong stimulating effect on growth of established lung metastases. The results indicate that blood loss may be an important factor in determining the outcome of metastatic growth.

Introduction

Blood transfusions appear to have numerous consequences which were never envisaged at the outset. In spite of careful blood donor screening, a substantial number of patients receiving transfusions experience some type of serious adverse effect, **e.g.** serum hepatitis, Acquired Immune Deficiency Syndrome, sensitization to foreign antigens. A well recognized beneficial effect is the longer survival of kidney grafts in recipients given blood transfusions prior to transplantation; this has also been confirmed in various animal models [1-3]. There is no agreement concerning the mechanism(s) responsible for this phenomenon, but the modulation of the host's immune response by blood transfusions has generally been implicated i.e. suppressor cell induction; anti-idiotypic antibodies; non-specific immunosuppression [4-6]. Recent clinical retrospective studies have demonstrated an association between perioperative blood transfusions and tumor recurrence in surgically treated patients [7-10]. These findings are also supported by the results of experimental work in rats carried out by Francis et al[11] and ourselves. We have demonstrated a promoting effect of blood transfusion on growth of artificial metastases[12].

Several disorders in the immune system have been reported following surgical trauma. Riddle et al [13] have shown a decline in lymphocyte response to Phytohaemagglutinin in postoperative patients; Constantian et al[14] demonstrared the presence of an endogenous immunosuppressive polypeptide in serum of traumatized patients; Slade et al[15] have demonstrated a decrease in a multitude of immune parameters measured in vivo and in vitro in normal patients following nephrectomy. In cancer patients, it is often the case that the only curative treatment is surgical resection of the primary tumor. This is usually an extensive surgical procedure with profound blood loss, often involving subsequent blood transfusions. The results mentioned above on immunomodulation following blood transfusions and surgery, together with the retrospective data indicating a decreased survival rate in patients receiving blood transfusion and undergoing surgical procedures, indicate grave consequences for cancer patients.

A possible solution might be to reduce the need for allogeneic blood transfusions, and thus avoid the subsequent effects on the patient's immune apparatus. The use of autologous blood transfusions would circumvent this hazard. This would involve collecting autologous blood from the patients prior to surgery. The technical feasibility of this procedure has already been demonstrated [16-17]. The consequences for the immune status of cancer patients of pre- and peroperative blood loss have, however, not yet been investigated.

In the present communication we report on the effects of blood loss, surgery and subsequent blood transfusions on the growth of established tumor metastases, using a rat model. Furthermore, the effect of blood loss alone and in combination with blood transfusions on the "take" of tumor cells was studied, since this might be of consequence for the tumor cells released in patients due to manipulation of the tumor during surgery.

Material and methods

Animals: Male rats of the inbred BN and WAG strains were used. The animals, aged 16-20 weeks, were bred under specific pathogen-free conditions.

Tumor: Tumor LS 175 is a spontaneous, non-immunogenic sarcoma in BN rats. The tumor is maintained as a stationary culture in Dulbecco's minimum essential medium, supplemented with 10% fetal calf serum (FCS). To obtain cells for the in vivo experiments, free floating LS175 cell clumps were harvested from the tissue culture flasks and, after washing, were resuspended in Hank's balanced salt solution (HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and was between 90 and 95%.

Lung colonies assay: 2.5×10^5 tumor cells, suspended in a volume of 1 ml, were injected intravenously into experimental and control rats. The number of colonies developing in the lungs were counted after 27 days. The lungs were excised, rinsed in tap water and subsequently fixed in Bouin's solution. Tumor nodules on the lung surface, visible to the naked eye, were counted.

Blood transfusions: BN rats received a single i.v. injection of 1 ml heparinized whole WAG blood or 1 ml of syngeneic blood. Control animals received no transfusions.

Bood loss: A volume of 4 ml blood was bled from BN rats by means of a tail incision. This volume represents approximately 30% of the total blood volume of the rats used.

Adominal surgery: Under ether anesthesia, an ileum resection at a length of 5-6 cm was performed, followed by reanastomoses using continuous suturing with 7.0 silk was performed. The procedure lasted 15-20 minutes, with an estimated blood loss of ± 2 ml.

Satistical analysis: All data were analysed by two way testing according to "Mann-Whitney" test when comparing two groups; and employing "Kruskal-Wallis" test for simultaneous comparison of more then two groups. The experimental error rate was reduced by using the significance level of $P < 0.01$ [18].

Experimental design

To investigate the effect on "Take" of tumor cells, animals were bled 7 days prior to tumor cell inoculation and subsequently given allogeneic or syngeneic blood transfusions on the day of tumor inoculation. To study the effect on "growth" of established lung metastases animals were bled 7 days after the tumor cell inoculation. On Day +14, animals received syngeneic or allogeneic blood

large standard deviations, both were found to be significantly higher than the control group; $P < 0.005$ and $P < 0.0001$ respectively. There was no statistically significant difference between the 3 experimental groups. These results indicate a substantial stimulation of growth of established lung metastases following blood loss.

Effects of bleeding, abdominal surgery and blood transfusions on growth of lung metastases.

BN rats injected with tumor cells on Day 0 were bled on Day +7. On Day +14, groups underwent procedures as shown in Table 3; **i.e.** they received syngeneic or allogeneic blood transfusions; underwent abdominal surgery or additionally received blood transfusion. The control group was only inoculated with tumor cells on Day 0. The results are presented in Table 3. The groups which were bled only, or additionally received syngeneic or allogeneic blood transfusions, showed a significantly higher number of lung colonies, as compared to the control group ($P < 0.0002$). This is in accordance with the results shown in Table 2. Furthermore, the number of colonies were significantly higher in the group receiving allogeneic transfusion in comparison to syngeneically transfused and the group of animals which were only bled. This illustrates the determental effect of blood transfusion superimposed on the effect of blood loss. The mean number of colonies of the group bled on Day +7 and undergoing abdominal surgery on Day +14 was 76 ± 26, which is significantly higher than the mean of the groups bled, undergoing abdominal surgery and additionally receiving syngeneic or allogeneic blood transfusions had a mean number of colonies of 135 ± 53 and 179 ± 50 respectively, which is significantly higher than the control group. These results demonstrate stimulation of growth of lung metastases following blood loss alone, or in combination with blood transfusions and/or abdominal surgery.

Discussion

Abraham et al [19, 20] reported a profound depression of inflammatory response and decreased mitogenic induced lymphocyte proliferation, following hemorrhage. Loegering et al[21] reported deficiency of circulating opsonic activity and reticulo-endothelial (RE) system depression during hemorrhagic shock. Also it is a well recognized fact in the clinic [22] that patients are susceptible to sepsis following trauma and hemorrhage. When entertaining the idea of autologous blood transfusion, bleeding the cancer patients prior to surgery would be a prerequisite. Considering the reports above, this prerequisite could have consequences for the "Take" of tumor cells released during manipulation of the primary tumor during surgery. The data in Table 1 show that blood loss on Day -7 did not have an effect on the "Take" of tumor cells on Day 0. Subsequent syngeneic or allogeneic blood transfusions following blood loss also had no effect on "Take". In this tumor model, we have shown previously [12]

that blood transfusions on Day -7 had no effect on "Take" of tumor cells. A possible explanation could be that in the process of "Take" factors other than the immune status are of greater importance. These could be coagulation status at time of tumor inoculation; inter-tumor cell interaction; etc. The data presented in Table 2 demonstrate a stimulating effect of blood loss on the growth of established metastases. We have shown previously [12], that syngeneic blood transfusion does not affect the growth of established lung metastases in this tumor model. However, the present results show a stimulating effect in the group receiving syngeneic blood transfusion when combined with prior blood loss. A similar effect is also observed in the animals which were only bled. This indicates an overwhelming adverse effect of blood loss. The group which was given allogeneic blood also showed greatly enhanced growth of lung colonies, and these results are confirmed by the results in Table 3. To our knowledge the effect of blood loss, as stated above, has not previously been investigated. Hattari et al[23] have demonstrated enhancing effects on tumor growth and Jubert et al[24] have shown impairment of immunocompetence, following peroperative blood loss. Our results show a similar trend: an enhancing effect on tumor growth is observed in groups undergoing abdominal surgery and additionally receiving allogeneic or syngeneic blood transfusions, preceded by blood loss on Day +7. An explanation for discrepancy between the effects of blood loss on "Take" and growth of established metastases could be the importance of different factors exhibiting a prominent role in the different phases of tumor kinetics. The assumed immunosuppresion produced by blood loss, blood transfusions and surgery might be responsible for the enhanced growth of metastases; while other factors, as stated above, play an eminent role in the outcome of "Take" of tumor cells. These results stress the need for further investigations of immunoparameters following a period of blood loss.

Pre-operative blood donation for autologous blood transfusion is a very plausible solution for the reported detrimental effect of blood transfusion on survival rates of cancer patients undergoing surgery. Further investigations should certainly be carried out, but as shown in our tumor model the prerequisite for any autologous blood transfusion **i.e.** blood loss prior to surgery, may have serious consequences.

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CHAPTER 10

ABROGATION OF THE TUMOR PROMOTING EFFECT OF ALLOGENEIC BLOOD TRANSFUSION BY POLYADENYLIC-POLYURIDYLIC ACID(POLY A-POL Y U)

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Summary

Surgical procedures have been shown to have an immunosuppressive effect. Retrospective studies have shown blood transfusions to be associated with an adverse prognosis of surgically treated cancer patients. We have previously demonstrated an enhanced growth of tumor metastases,in rats, following allogeneic blood transfusion and surgery. Poly A-poly U has been reported to stimulate immune responses. In this report, we have investigated the effectiveness of poly A-poly U as an adjuvant to blood transfusion and surgical procedures in BN rats bearing artificial lung metastases. Significantly reduced tumor growth was observed following poly A-poly U adjuvant treatment.

Introduction

The current therapeutic approach to treatment of cancer patients is multi-disciplinary. Surgical, hormonal, immune and radio-chemo-therapy play the major role. Many studies have indicated an immuno-suppressive effect of several treatment modalities. Stratton et al [1] have shown a profound effect of irradiation on lymphocyte subpopulations in cancer patients. Various chemotherapeutic regimes are known to produce an immunosuppressive effect[2]. Numerous reports have indicated the most commonly employed therapy for cancer patients, **i.e.:** surgical resection, to have an immunosuppressive effect[3,4]. Anesthesia, the inevitable accompaniment of a surgical procedure, is also shown to have similar effects on the immune apparatus[5]. Furthermore, surgery in cancer patients is often accompanied by substantial blood loss, necessitating administration of a blood transfusion. Blood loss on its own has been shown to produce decreased immuno-competence [6,7]. Several recent retrospective studies have indicated a relationship between blood transfusions and adverse prognosis of disease-free survival of surgically-treated cancer patients [8,9] and has been subsequently been confirmed in animal studies[10,11]. In a rat model, we previously demonstrated a detrimental effect of surgery in combination with allogeneic blood transfusion on the growth of established artifical tumor metastases[12,13]. Surgical treatment of cancer patients may result in marked immunosuppression and eventual enhancement of tumor metastatic growth.

A double-stranded synthetic polynucleotide, polyadenylic-polyuridylic acid (poly A - poly U), has been reported to stimulate immune responses. It has been shown to possess an anti-tumor effect, and to be a potent immune modulator of both the humoral and cellular immune response and to result in enhancement of natural killer cell activity (NK cell)[14-18]. Furthermore, no toxic or pyrogenic effects of poly A - poly U have been reported even when used at high dosage, as adjunct in cancer therapy[19,20].

Previously, we have shown an enhanced growth of LS 175 tumor metastases in BN rats undergoing abdominal surgery in combination with allogeneic blood transfusion. Enhanced metastases growth is also observed following allogeneic blood transfusion alone[11-13]. This enhanced growth is found to be correlated with decreased T-cell activity (chapter 7). In the present manuscript we report on the effect of adjuvant treatment with poly A-poly U in tumor-bearing rats undergoing abdominal surgery and treated with blood transfusions.

Material and methods

Animals: Male rats of the inbred BN and WAG strains were used. The animals, aged 16-20 weeks, were bred under specific pathogen free conditions.

Tumor: Tumor LS 175 is a spontaneous, non-immunogenic sarcoma in BN rats. The tumor is maintained as a stationary culture in Dulbecca's minimum essential medium, supplemented with 10% fetal calf serum (FCS). To obtain cells for the

vivo experiments, free-floating LS 175 cell clumps were harvested from the tissue culture flasks and, after washing, were resuspended in Hank's balanced salt solution (HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and was between 90 and 95%.

Lung colonies assay: 2.5 X 10^5 tumor cells, suspended in a volume of 1 ml, were injected intravenously into experimental and control rats. The number of colonies developing on the lungs were counted after 24 days. The lungs were excised, rinsed in tap water and subsequently fixed in Bouin's solution. Tumor nodules on the lung surface, visible to the naked eye, were counted.

Blood transfusions: BN rats received a single i.v. injection of 1 ml heparinized whole allogeneic blood or 1 ml of syngeneic blood. Control animals received no transfusions.

Abdominal surgery: Under ether anesthesia a resection of a 5-6 cm segment of ileum was performed followed by end-to-end anastomoses using continous suturing with 7.0 silk. The procedure lasted 15-20 minutes, with an estimated blood loss of ±2ml.

Poly A -poly U: Poly A-poly U (Sigma P3259) powder was dissolved in phosphate buffered saline (PBS) and adjusted to a concentration of 3000μg/ml. 0.5 ml **i.e.:** 1500μg, of this solution was administered intravenously (i.v.) to experimental animals on days +7, +14 and +21.

Experimental design

The effectiveness of poly A-poly U in abrogating the adverse effect of allogeneic blood transfusion alone or in combination with surgery, on the growth of established lung metastases was investigated. BN rats were inoculated with 2.5 X 10^5 LS 175 tumor cells on day 0. The control animals did not receive any blood transfusion or poly A-poly U and did not undergo abdominal surgery. On day +7, experimental groups of animals received syngeneic or allogeneic blood transfusion alone or in combination with abdominal surgery. A group of rats underwent abdominal surgery but received no blood transfusion and another group was administered poly A-poly U alone without undergoing surgery or blood transfusion. All experimental groups were administered poly A-poly U in a dosage of 1500μg/week, on days +7, +14 and +21. Animals were sacrificed on day +24 and lung colonies were counted. Each group comprised 9-15 animals. The results were statistically analysed by means of the Student "t" test.

Table 1: Effect of poly A - poly U as adjuvant therapy on tumor growth.

Treatment on day + 7	**No. of animals**	**Mean no. of lung colonies (± S.D.)**	*
No treatment	15	60 ± 14	
Poly A-Poly U only	10	39 ± 7	S
Syngeneic transfusion + Poly A-Poly U	10	20 ± 4	S
Allogeneic transfusion + Poly A-Poly U	10	33 ± 10	S
Surgery + Poly A-Poly U	9	26 ± 6	S
Surgery + Syngeneic transfusion + Poly A-Poly U	9	40 ± 9	S
Surgery + Allogeneic transfusion + Poly A-Poly U	9	28 ± 4	S

poly A - poly U administered in a doses of 1500μg/wk on days +7; +14; and +21 after tumor inoculation.
* : Significantly different as compared to the control group.

Results

On day +24 the mean number of colonies of the control group, **i.e.:** animals inoculated with tumor cells on day 0 not undergoing any further experimental procedure, was 60 ± 14 (Table 1). All other experimental groups were treated with poly A-poly U on days +7, +14 and +21. Treatment with poly A - poly U alone resulted in 39 ± 7 lung colonies, which is significantly less than the control group. Syngeneic and allogeneic blood transfusion combined with poly A-poly U resulted in 20 ± 4 and 33 ± 10 lung colonies, respectively. These numbers were significantlylower than the control group. Surgery alone or in combination with blood transfusion and adjuvant poly A-poly U treatment also resulted in a significantly lower mean number of lung colonies. Surgery alone resulted in a mean number of lung colonies of 26+6, while in combination with syngeneic and allogeneic transfusion 40 ± 9 and 28 ± 4 lung colonies were found, respectively. These results indicate a profound suppressive effect on the growth of established lung tumor metastases following adjuvant treatment with poly A-poly U, even when the animals underwent surgery and received allogeneic blood transfusion.

Discussion

Lundy et al[21] have shown that the use of Thiobendazole, an immuno-restorative drug, as immunotherapy can be an effective adjuvant treatment to surgery in preventing the growth of micro-metastic foci. Lacour et al[22] have demonstrated a significant benefit of adjuvant treatment with poly A-poly U in operable breast cancer patients, demonstrating an increase in both overall and relapse free survival, particularly in the group with positive axillary nodes. Bonadonna et al[23] have reported similar success in treating breast cancer patients with CMF (cyclophasphamide, methotrexate and flourouracil) as adjuvant therapy. However, in contrast to poly A-poly U, several side-effects following CMF use were reported. The results of this study confirm the beneficial use of poly A-poly U, a immuno-stimulatory drug, as adjuvant in cancer therapy. Previously, in the same tumor-host model employed in this study, we have demonstrated a significant enhancement of tumor growth following allogeneic blood transfusion alone or in combination with abdominal surgery [11-13]. These animals also showed a significantly diminished mitogen blastogenic response to Con A and PHA stimulation. In the present study, poly A-poly U is shown to be effective in abrogating the adverse effect of blood transfusion and surgery on tumor growth. In the same tumor-host model, poly A-poly U treatment was found to result in a significant stimulation of mitogen blastogenic response and gamma-interferon production capacity[18]. Youn et al[24] have shown a significant enhancement of NK cell activity following poly A-poly U treatment of operable stomach cancer patients. The anti-tumor and the stimulating effect on cellular immune response, NK cell activity and interferon production capacity of poly A-poly U treatment could be the mechanisms responsible for offsetting the detrimental effects of surgery and blood transfusions, and resulting in a significantly diminished growth of tumor metastases [14-16,25].

Surgical resection is still the most frequent therapy employed in treatment of cancer patient. In spite of improved surgical techniques, perioperative blood loss often necessitates the administration of a blood transfusion. Recent retrospective studies indicate an adverse effect of blood transfusion on disease-free survival[8, 9]. This blood transfusion effect could lead to restrictions in the surgical procedures employed in treating cancer patients. Thus, one should consider the use of immuno-restorative adjuvant therapy in surgical treatment, especially when blood transfusions are also administered. The absence of any reported toxic, pyrogenic or other secondary effect of poly A-poly U and its demonstrated effective employment as adjuvant chemotherapy agent in clinical studies, makes poly A-poly U a strong candidate for use as described[19]. The results of the present study and other experimental studies further substantiate the effectiveness of its use.

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CHAPTER 11

GENERAL DISCUSSION

The present study demonstrates that allogeneic blood transfusions are capable of modulating different processes of tumor progression. In two different tumor-rat models varied effects were observed: blood transfusion in one model was found to inhibit the take but have no effect on growth of established artificial tumor metastases; whereas in the other, transfusion had no effect on take but strongly stimulated the growth of the metastases(Table 1). Erythrocyte and leukocyte transfusates were as effective as whole blood in stimulating growth, whereas plasma transfusion failed to have an effect[chapter 4,6].

Table 1: Summary.

Experimental procedure	Tumor	Tumor-take	Tumor-growth
Allogeneic blood transfusion	Tumor LS 175	No effect	Stimulation
Allogeneic blood transfusion	Tumor BC 1618	Inhibition	No effect
Surgery	Tumor LS 175	Stimulation	No effect
Allogeneic blood transfusion + Surgery	Tumor LS 175		Stimulation
Allogeneic erythrocytes transfusion	Tumor LS 175		Stimulation
Allogeneic leukocytes transfusion	Tumor LS 175		Stimulation
Allogeneic plasma transfusion	Tumor LS 175		No effect

These diverse effects of blood transfusion are similar to the results found in clinical retrospective studies **ie:** some studies find a correlation between poor prognosis of surgically treated cancer patients and blood transfusions, while other studies fail to confirm such a relationship [chapter 2]. A comparative array of effects is found in studies on the effect of blood transfusions on prognosis of an organ transplant, in clinical and experimental animal studies. A prolonged graft survival is reported in patients, and in animal models, transfused prior to transplantation[1,2], whereas various studies have reported no beneficial effect using donor-specific or third party blood transfusions[3,4]. In the WAG to BN heart transplantation model, a **donor specific** transfusion results in sensitization, reflected in accelerated graft rejection. In our studies, however, a WAG allogeneic blood transfusion to BN rats was found to stimulate syngeneic tumor growth, which would seem to contradict the immunostimulatory effect observed in the organ graft model. However, recent preliminary results indicate that a blood

transfusion, parallel to donor specific sensitization can induce a degree of aspecific immunosuppression, as reflected in prolonged survival of a third party organ graft (Singh, unpublished data). This indicates the existence of paradoxical specific and non-specific immunological effects of blood transfusion in the same blood recipient at the same time. Furthermore, as opposed to the situation in the transplantation model, it is the effect of a third party blood transfusion which is observed in the tumor growth model. Some authors have put forward that immunosuppression in a transplantation model following blood transfusion is the result of leukocytes in the transfusate. However, the merits if this argument cannot be investigated, since there is always a degree of leukocyte contamination, irrespective of the method used for purification. Furthermore, we [chapter 6] and Oikawa et al[5] have demonstrated that the effect of blood transfusion as observed on tumor growth can be elicitated by administration of erythrocytes and leukocytes alone. Thus, the detrimental effect of transfusion on tumor growth could not be avoided by employing a leukocyte-free erythrocyte transfusion.

In accordance with the results of organ transplantation models, the blood transfusion effect on tumor growth is found to be dependent on several factors **i.e.:** the tumor employed, and the blood donor-recipient combination. Jeekel et al., employing WAG rats bearing a transplantable adeno-carcinoma, reported inhibition of tumor growth after a BN rat whole blood transfusion, as opposed to no effect in WAG rats on tumor BC 1618 observed in our studies[5]. Other experimental studies, investigating this blood transfusion phenomenon, also demonstrate a similar variety of effects on tumor growth. Francis et al., in a rat tumor model, have reported an enhanced tumor growth following an allogeneic transfusion[6]. In other tumor models Oikawa et al., Zeller et al. and Nathanson et al. have shown effects ranging from inhibitory to no effect on tumor growth[7-9].

Factors other than blood transfusion could be the culprits responsible for the poor prognosis of surgically-treated patients found in the retrospective studies. In our study, we found no effect of anesthesia on tumor growth. However, a profound enhancing effect of abdominal surgery on the take of tumor cells was demonstrated [chapter 5]. This could be of great consequence for the take of tumor cells released by tumor manipulation during surgical tumor resection. To avoid such an effect, the no-touch technique, though not proven to lead to a decreased number of metastases, might be recommended as the surgical procedure of choice.

Eggermont et al., in a mice-tumor model, have shown an augmentation of tumor growth following a surgical procedure[10]. In the BN-LS 175 tumor model, abdominal surgery was found to have no effect on the growth of established metastases. However, strong stimulation of metastatic growth was observed after abdominal surgery in combination with allogeneic blood transfusion. Whether this effect is evoked by allogeneic blood transfusion alone or amplified by the surgical stress, is open to speculation.

The difference in effects of blood transfusion, surgery alone or surgery

combined with transfusion, on different stages of tumor progression may be explained by the relative importance of different host and tumor factors during various stages. NK cell activity is believed to be of consequence for the take of tumor cells, while being less effective on the growth of established metastases[11]. However, a significant increase in NK cell activity was observed after abdominal surgery, which was found to enhance, rather than inhibit, the take of tumor cells [chapter 5,7]. This indicates that factors other than NK cells may be of crucial importance. In the results reported in chapter 7, a correlation was found between enhanced tumor growth and a decrease in T-cell mitogen stimulation response, following allogeneic blood transfusion alone or in combination with surgery. Francis et al. and Nathanson et al. have also shown a similar depression of T-cell activity after allogeneic blood transfusion[5,7]. Acceleration of tumor growth was also observed following cyclosporin treatment (chapter 7), a drug believed to specifically inhibit T-cell function, providing indirect evidence of susceptibility of the tumor to T-cells. Furthermore, poly A-poly U, an immunomodulator capable of enhancing the cellular immune response, was found to be effective in eliminating the detrimental effect of allogeneic blood transfusion [chapter 10]. These results indicate a certain role of T-cells in the blood transfusion phenomenon. However, the tumor used in our studies, LS 175, is known to be non-immunogenic and thus would not be expected to be affected by changes in the host's T-cell dependent immune status. In spite of enormous growth in the number of publications each year, our knowledge of non-immunogenic tumors, a category to which most human cancers belong, is extremely rudimentary. Most of the reports in the literature use a chemically or virally-induced immunogenic tumor. In a random selection of papers published in one year, Hewitt et al.[12] found that in less than 7% of the cases a spontaneous tumor, which is mostly non-immunogenic, was used. Because of this lack of knowledge about the manner of interaction between an non-immunogenic tumor and the immune apparatus of the host, no classical explanation for the possible correlation between T-cell function and growth of LS 175 can be presented. It is generally assumed that so called non-immunogenic tumors are not capable of inducing specific immunity which influences the growth of the tumor after immunization. However, a degree of specific control may be exercised by the immune apparatus, in spite of the measure of tumor growth observed. Disturbance of this equilibrium between immunological control and tumor growth, may lead to modulation of tumor growth. Although the above hypothesis is speculative, a similar equilibrium is believed to be functional for the dormant cancer cell, a phenomenon in which recurrence of cancer may occur after a latent period of up to 30 years[12,13]. Furthermore, the classification immunogenic versus non-immunogenic is dependent on the tools employed during this procedure. The use of other or more sensitive assays could lead to a different classification of the same tumor.

Factors not related to T-cells may be of importance in precipitation of the blood transfusion phenomenon. Rather than immunological factors, polypeptide hormones and hormone like growth factors may be the prominent agents in the

tumor growth modulation observed. Malignant cells have been shown to be capable of producing growth factors which stimulate their own growth, thus rendering them independent of serum factors[14]. This property of cancer cells may be of crucial importance in the growth of established tumor metastases. These mediators are often only produced locally and in extremely small amounts, and vanish rapidly. We were unable to detect tumor growth factors after blood transfusion and surgery. However, our assay is a reflection of the factors present in peripheral blood, whereas factors obtained from the local enviroment of metastases could be more representive for the status assay. Similarly, the immune parameters were determined in peripheral blood and spleen cells; these may not correlate intimately with the immune status in and around the tumor metastases. The evolution of techniques of immunohistochemistry and monoclonal antibodies at present allow the identification of various immunological cells and their functional subsets. Employment of such methods in evaluating populations of cells and various growth mediators in and around the immediate vicinity of tumor cells could provide a better understanding of the role of different factors in tumor biology.

In recent years, much attention has been focused on prostaglandins(PGs). T-lymphocytes, macrophages and tumor cell are all capable of PG production, especially prostaglandins E (PGE). A marked increase of PG production is reported in malignant tumors[15,16]. Kort et al., using the BN-LS 175 model, the same combination as employed in our studies, have demonstrated high plasma tromboxane B_2 and PGE_2 levels with growth of the tumor, and decrease of metastatic growth with aspirine therapy[17,18]. Recently, a significant increase in prostaglandin E production after blood transfusion has been reported [19-21]. Furthermore, Shelby et al. have shown that the blood transfusion induced suppression of a graft-vs-host response in rats was abrogated by concomitant treatment with indomethacin[22]. If these observations are taken together they indicate a possible role for the PGs in the mechanism of the blood transfusion phenomenon in relation to cancer growth. Investigations to elucidate this speculation may provide clues as to the enigma of the blood transfusion phenomenon.

Investigations reported in this thesis demonstrate that allogeneic blood transfusion can result in enhanced tumor growth. However, the results of an ongoing prospective clinical trial on the effect of blood transfusions on the outcome of colon cancer patients, in Rotterdam, should provide the ultimate answer to the question whether the transfusion phenomenon demonstrated in our study is an artefact or reality in the clinical setting.

Finally, while the blood transfusion may be inevitable, the subsequent administration of immune-modulators **e.g.:** poly A-poly U may help to counteract its detrimental effect. This approach requires further investigation.

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CHAPTER 12

SUMMARY

In the course of their treatment, cancer patients frequently require blood transfusions. In the field of transplantation, blood transfusions have been demonstrated to result in prolonged organ graft survival. The proposed existence of an analogy between the antigenicity of an organ graft and a tumor raises the question of a possible relationship between tumor growth and blood transfusion. The experiments described in this thesis investigated various aspects of surgical treatment of a cancer patient, including blood transfusions, on tumor behaviour in an experimental model. A short review of the relevant aspects of immunology with respect to tumors, as they may relate to blood transfusions, is presented in chapter 1. Chapter 2 furnishes the detailed arguments which form the basis of the experiments in this thesis. Results of clinical and experimental investigations demonstrate an array of effects ranging from inhibition to no effect to stimulation of tumor growth following blood transfusion. Clarifying remarks, thought to be helpful in the interpretation of the results reported in the thesis, are made in chapter 3.

Allogeneic blood transfusion was found to be capable of modulating tumor growth in two different tumor models in rats(chapter 4). A single blood transfusion was found to stimulate the growth of tumor LS 175, a non-immunogenic sarcoma transplantable in BN rats, but had no effect on the take of the tumor. However, in the BC 1618 tumor model, a highly immunogenic basal cell carcinoma, transplantable in WAG rats, no effect on growth and inhibition of the take of the tumor was observed. This demonstrates that the mode of tumor growth modulation following transfusion is dependent on the tumor and host employed and the aspect of tumor kinetics investigated. An effect on tumor growth was assumed to have been observed if blood transfusion was administered after tumor inoculation, the take of the tumor was assumed to be modified if transfusion was given before tumor injection.

Investigations into other aspects of surgical treatment were carried out by employing a tumor-host model in which transfusion resulted in enhanced tumor growth, i.e.: the LS 175 model in BN rats. In this model BN rats recieved 2.5 x 10^5 tumor cells i.v. leading to the development of artificial lung metastases, and then underwent the experimental procedure under study. The number of lung metastases counted four weeks after tumor inoculation was the final assay of the experiments. Neither an ether anesthesia nor surgery alone was found to have an effect on the growth of tumor LS 175. However, abdominal surgery in conjunction with allogeneic blood transfusion significantly increased tumor growth (chapter 5). Furthermore, surgery itself was found to increase greatly the take of the tumor cells.

Some authors claim that the blood transfusion effect in organ transplantation is the result of the presence of leukocytes in the transfusate. Results in support as well as against this school of thought can be found in the literature. This led us to investigate the role of different blood constituents on tumor growth. Erythrocytes and leukocytes were found to be capable of stimulating tumor growth, similar to the effect of whole allogeneic blood. However, a plasma transfusion was found to have no effect (chapter 6).

One of the reasons for the administration of blood transfusions to a cancer patient undergoing surgery is pre-, per- or post-operative blood loss. The effect of blood loss on tumor growth has not been appropriately investigated and so we looked at its consequences in the LS 175 tumor model. We found that a blood loss of approximately 30% of the total blood volume, alone or in combination with surgery and blood transfusion, strongly stimulated the growth of artificial lung metastases (chapter 9). Thus, in the clinical retrospective studies, reporting on the effect of transfusion on tumor growth, blood loss may have been an important factor which was not taken into consideration.

The positive effect of transfusion on organ graft survival is thought to be due to an immunosuppressive effect of transfusions. However, the mechanism underlying this is not yet known. We have investigated the possible role of the immune system in the blood transfusion effect in relation to cancer (chapter 7,8). An allogeneic blood transfusion was found to result in a decreased T-cell activity, which may reflect a mechanism precipitating the blood transfusion phenomenon. However, following surgery and transfusion the natural killer cell and the leukocyte phagocytic activity significantly increased. This increase did not correlate with the observed tumor growth and did not appear to have a role in modulation of tumor behavior (chapter 7). Furthermore, an immune modulator, Poly A- Poly U, was found to be successful in abrogating the detrimental effect of blood transfusion on tumor growth. This provides further credence for the blood transfusion phenomenon, as observed here, to be an immunological phenomenon.

Factors not related to immunology may also be of importance. We attempted to find a possible role of growth factors in the transfusion phenomenon, but were unsuccessful. Finally, other possible mechanisms and future consequences of the blood transfusion phenomenon reported here are discussed in chapter 11.

CHAPTER 13

SAMENVATTING

Kankerpatienten die voor hun tumor worden geopereerd krijgen vaak bloedtransfusies. Uit de transplantatiebiologie is bekend dat bloedtransfusies immunosuppressie veroorzaken en de acceptatie van orgaantransplantaten verbeteren. Indien men de hypothese aanhangt dat een tumor, net zoals een transplantaat, immunogeen is voor zijn gastheer, dringt zich de vraag op of bij kankerpatienten bloedtransfusies de groei van tumoren zouden kunnen beinvloeden.

Behalve bloedtransfusies zijn er ook andere aspecten rond een operatie die mogelijk van invloed zijn op het gedrag van een tumor; anesthesie, de chirurgische ingreep zelf en bloedverlies. De experimenten die in dit proefschrift beschreven staan hadden tot doel inzicht te verschaffen in de afzonderlijke betekenis van deze aspecten voor de groei van tumoren. Voor het onderzoek werd gebruik gemaakt van twee tumormodellen bij de rat.

In hoofdstuk 1 wordt een overzicht gegeven van de recente inzichten in de tumorimmunologie. In hoofdstuk 2 staan de experimentele en klinische resultaten vermeld die de hypothese dat er een verband zou zijn tussen bloedtransfusies en tumorgroei steunen dan wel omver werpen. Hoofdstuk 3 geeft een nadere toelichting op de in dit proefschrift gebruikte tumormodellen, waardoor de interpretatie van de behaalde resultaten meer inzichtelijk wordt.

In hoofdstuk 4 staan experimenten beschreven waarin het effect van bloedtransfusies in twee tumormodellen werd bestudeerd. In het eerste model werd gebruik gemaakt van een niet-immunogeen sarcoom bij ingeteelde BN ratten. Allogene transfusies gaven aanleiding tot een versnelde groei van deze tumor, maar hadden geen invloed op het aanslaan van (artificiele) metastases. Het tweede model bestond uit een sterk immunogene huidtumor bij ingeteelde WAG ratten. De groei van deze tumor werd niet door allogene transfusies beinvloed, echter het aanslaan van longmetastasen werd door transfusies aanzienlijk geremd. Deze sterk wisselende resultaten lijken aan te geven dat het transfusie-effect wordt bepaald door zowel factoren in de tumor als in de gastheer. Het eerste tumor model werd verder aangewend om de rol van chirurgie en ether anesthesie, al of niet in combinatie met bloedtransfusies te bestuderen (hoofdstuk 5). De procedure was als volgt: BN ratten kregen $2.5x10^5$ sarcoomcellen intraveneus toegediend wat leidt tot de ontwikkeling van longmetastasen die na 4 weken goed te tellen zijn. De te onderzoeken variabelen (transfusie, chirurgie, anesthesie) werden voor of na de inoculatie van tumorcellen toegediend, zodat de invloed op respectievelijk het aanslaan dan wel de uitgroei van de tumor kon worden bestudeerd. Ether anesthesie en chirurgie, welke bestond uit het verwijderen van een stukje dunne darm, hadden geen invloed op de groei van gevestigde

longmetastases. Echter, chirurgie gecombineerd met een allogene bloed transfusie had weer een sterk stimulerend effect. Het aanslaan van de tumor werd aanzienlijk bevorderd door chirurgie.

In hoofdstuk 6 worden experimenten beschreven die tot doel hadden de invloed van verschillende bloedcomponenten op tumorgroei te bestuderen. Gezuiverde leukocyten en erythrocyten suspensies waren beide in staat het bloedtransfusie fenomeen te induceren terwijl plasma geen effect had.

Een belangrijke reden om peroperatief bloed aan kankerpatienten toe te dienen is bloedverlies. Over het effect van bloedverlies op tumorgedrag is weinig bekend, reden om dit te bestuderen in ons tumormodel. Er werd gevonden dat verlies van ongeveer 30% van het totale bloedvolume de groei van longmetastases sterk stimuleerde, ook indien dit werd gecombineerd met chirurgie of bloedtransfusies (hoofdstuk 9). Deze bevinding geeft aan dat bij de interpretatie van de retrospectieve klinische gegevens over de relatie bloedtransfusies- tumorgroei, ernstig rekening gehouden moet worden met de additionele rol van bloedverlies.

Het positieve effect dat bloedtransfusies hebben op de acceptatie van orgaan transplantaten wordt toegeschreven aan de inductie van immunosuppressie. Ook in ons tumormodel hebben we onderzocht of er een relatie bestond tussen het immuunsysteem en het groeigedrag van de tumor na bloedtransfusies en chirurgie (hoofdstukken 7 en 8). De belangrijkste bevinding was dat allogene transfusies resulteerden in een verminderde T-cel respons. De activiteit van NK-cellen en de fagocytose capaciteit van leukocyten daarentegen bleken verhoogd na bloedtransfusies en chirurgie. In aanmerking genomen dat het sarcoom dat werd gebruikt tijdens deze studie niet-immunogeen is en dientengevolge ongevoelig zou moeten zijn voor een klassieke T-cel depressie maar gevoelig voor modificaties in natuurlijke immuniteit, zijn de door ons gevonden immunologische bevindingen moeilijk in een begrijpelijk kader te vatten. De resultaten die staan vermeld in hoofdstuk10, en handelen over het vermogen van de immunomodulator poly A-poly U om het transfusie effect teniet te doen, doen eveneens vermoeden dat het immuunsysteem op onbegrepen wijze betrokken is bij de beschreven fenomenen.

In hoofstuk 11 wordt nader ingegaan op het mogelijke mechanisme van het transfusie-effect en wordt aangegeven dat prostaglandines en groeifactoren mede van belang zouden kunnen zijn.

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